FRESHWATER CYANOTOXIN MIXTURES IN RECURRING CYANOBACTERIAL BLOOMS IN VOYAGEURS NATIONAL PARK

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Program:
Environmental and Conservation Science

May 2021

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Algal and cyanobacterial blooms can foul water systems, inhibit recreation, and produce cyanotoxins, which can be toxic to humans, domestic animals, and wildlife. Blooms that recur yearly present a special challenge, in that chronic effects of most cyanotoxins are unknown. To better understand cyanotoxin timing, possible environmental triggers, and inter-relations among taxa and toxins in bloom communities, recurring cyanobacterial blooms were investigated at three recreational sites in Kabetogama Lake in Voyageurs National Park from 2016-2019. Results indicated that peak neurotoxin concentrations occurred before peak microcystin concentrations and that toxin-forming cyanobacteria were present before visible blooms, which is a serious human health concern. Two cyanotoxin mixture models (MIX) and two microcystin (MC) models were developed using near-real-time environmental variables and additional comprehensive variables based on laboratory analyses. Comprehensive models explained more variability than the environmental models and neither MIX model was a better fit than the MC models. However, the MIX models produced no false negatives, indicating that all observations above human-health regulatory guidelines were simulated by the MIX models. The results show that a model based on a cyanotoxin mixture is more protective of human health than a model based on microcystin alone. In 2019, 7 of 19 toxins were detected in various mixtures. The potential toxin producing cyanobacteria, *Microcystis*, was significantly correlated with microcystin-YR, while *Pseudanabaena* sp. and *Synechococcus* sp. were negatively correlated to several toxins. Jaccard and Sorenson indices indicated strong same-day similarities among the three bloom communities. Nitrogen-fixing cyanobacteria were present at every site, and when combined with internal loading of phosphorus, might explain similarities among sites, and why seasonal differences, even in samples from the same site, were stronger. Information from this

dissertation adds to the body of work on recurring blooms and under-studied toxins and toxin mixtures, providing a better understanding of future research options for freshwater cyanotoxins in and outside of Voyageurs National Park.

ACKNOWLEDGMENTS

I express sincere appreciation to my advisors and committee members, Dr. Eakalak Khan, Dr. Jack Norland, Dr. John McEvoy, and Dr. Zoltan Majdik. I also thank Ryan Maki, Jaime LeDuc, and Seth McWhorter, along with other collaborators at the National Park Service (NPS) for field assistance, logistical help, and brainstorming new research directions at Voyageurs National Park. A special thank you goes to my colleagues at the U.S. Geological Survey (USGS) who allowed me the freedom to follow this research direction and helped coordinate these and other research projects. These colleagues include Hayley Olds, Erin Stelzer, Dr. Jennifer Graham, Dr. Keith Loftin, and Barbara Eikenberry, in addition to Dr. Rebecca Kreiling and Dr. Karen Ryberg, who showed me it was possible to work full time and complete a PhD. I especially thank my colleague, Kathy Lee, for allowing me the freedom to work on this research. I recognize the following colleagues for peer review of the articles that resulted from this research, including Joe Duris, Dr. Jennifer Graham, Donna Francy, Jaime LeDuc, and Amanda Bell. I acknowledge these sources of funding for my salary and laboratory costs: the USGS-NPS Water-Quality Partnership Program and the USGS Environmental Health Program (Toxic Substances Hydrology and Contaminant Biology). Finally, I express gratitude to my late father, John Glenn, and my mother, Nancy Brown Glenn, who instilled in me the work ethic and sense of curiosity that makes science and research exciting. Thank you especially to my mother for her never-ending pride and encouragement in all my writing endeavors.

DEDICATION

Dedicated to my husband, Bryan, and my children Brooke, Jesse, and Claire. Thanks for sticking by my side and allowing me to slide a little on family obligations. I look forward to spending more quality time with all of you.

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AICc	Akaike's Information Criterion, corrected for small sample size
anaC	anatoxin-a synthetase C gene
AptA	anabaenopeptin A
AptB	anabaenopeptin B
AptF	anabaenopeptin F
BMAA	beta-N-methylamino-L-alanine
D	Secchi depth, in meters
dmLR	[Dha7]microcystin-LR
DNA	deoxyribonucleic acid
DO	dissolved oxygen
E	estimated value (occurs above the detection limit but below the quantification limit)
ELISA	enzyme linked immunosorbent assay
E. coli	*Escherichia coli*
EPA	U.S. Environmental Protection Agency
i.p.	intraperitoneal
L	liter
LD50	lethal dose for 50% of subjects
LL_14day	lake level change in feet over the last 14 days at 10 a.m. (including sampling day)
LL_7day	lake level change in feet over the last 7 days at 10 a.m. (including sampling day)
LL_Spring	the difference between lake level on the day of sampling and the spring average (April and May) lake level, in feet
LoD	limit of detection

LoQ .....limit of quantification

m .....meters

MC .....microcystin

MCLA .....microcystin-LA

MCLR .....microcystin-LR

MCYR.....microcystin-YR

mcyE.....microcystin synthetase gene

MDL.....method detection limit

mg/L .....milligrams per liter

Micro_mcyE .....*Microcystis*-specific microcystin toxin gene copies

MIX.....weighted concentration of anatoxin-a, microcystin, and saxitoxin

mL .....milliliters

MLR.....multiple linear regression

MRL .....minimum report level

MW .....molecular weight

µg/g .....micrograms per gram

µL.....microliters

µg/L.....micrograms per liter

µm .....micrometers

µS/cm .....microSiemens per centimeter at 25 degrees Celsius

N+N .....nitrite plus nitrate

N:P .....nitrogen to phosphorus ratio

NA.....not applicable

NC.....not calculated

ND.....not detected

ng/L.....nanograms per liter

NH_3.....ammonia in milligrams per liter as nitrogen

OP.....orthophosphorus

PAR.....photosynthetically active radiation

PC.....phycocyanin

PCR.....polymerase chain reaction

Plank_mcyE.....*Planktothrix*-specific microcystin toxin gene copies

ppb.....parts per billion

PRESS.....predicted residual sum of squares

PST.....paralytic shellfish toxin

qPCR.....quantitative polymerase chain reaction

RNA.....ribonucleic acid

RPD.....relative percentage difference

rRNA.....ribosomal ribonucleic acid

R^2.....coefficient of determination

RSD.....relative standard deviation

SC.....specific conductance

sxtA.....saxitoxin biosynthesis gene

TN.....total nitrogen

TP.....total phosphorus

USGS.....U.S. Geological Survey

UVB.....ultraviolet-B radiation

VB.....Virtual Beach software

VIF.....variance inflation factor

WHO .....World Health Organization

WindDir .....wind direction, instantaneous, in degrees

WT .....water temperature, in degrees Celsius

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CHAPTER 1: GENERAL INTRODUCTION

1.1. Background

Cyanobacteria, often referred to as blue-green algae, have had major effects on the Earth (Paerl and Paul, 2012), and not only in aquatic environments. They formed our oxygenated atmosphere, provide food for fish, and are important for multiple trophic levels. However, cyanobacteria can be harmful to ecosystems by contributing to hypoxia, disrupting the food web, and releasing toxins called cyanotoxins. Short-term human health responses to cyanotoxin exposure include rash, gastrointestinal symptoms, fever, and headaches (Hilborn et al., 2014). Cyanotoxin exposure has become a major concern, especially because cyanobacterial blooms tend to occur repeatedly in the same water supply (World Health Organization, 2003) and the long-term consequences of repeated exposure to low doses of cyanotoxins are unknown.

Cyanotoxins are classified into three main groups based on their target tissue: dermatoxins, hepatotoxins, and neurotoxins (Chorus and Bartram, 1999), which affect the skin, liver, and nervous system, respectively. Additional secondary metabolites produced by cyanobacteria, such as anabaenopeptins, are not typically classified as toxins, yet recent research indicates they may have detrimental effects on aquatic organisms (Lenz et al., 2019). The most frequently studied freshwater toxin is the hepatotoxin microcystin (Merel et al., 2013), whereas highly toxic neurotoxins, such as anatoxin-a and saxitoxin, have received less attention. Anatoxin-a has been linked to animal mortality (Heiskary et al., 2014; Sabart et al., 2015; Wood et al., 2007) and can cause death in minutes (Carmichael and Boyer, 2016). Saxitoxin is one of the most potent naturally occurring neurotoxins known (Cusick and Sayler, 2013; Loftin et al., 2016). Despite the potency of these neurotoxins, anatoxin-a and saxitoxin are understudied in fresh water.

Cyanobacteria are one of several types of phytoplankton, and changes in the water column often affect phytoplankton dynamics (Becker et al., 2010). For example, light and nutrient availability are often correlated with increased phytoplankton growth. Temperature increases can result in a decrease in the biomass of green algae (Becker et al., 2010), which may allow cyanobacteria to dominate in late summer. Cyanobacterial species presence, in turn, determines which toxins can be produced. Although there is abundant literature describing the temporal variation in light availability, nutrients, and phytoplankton succession (Feuchtmayr et al., 2010; Hecky and Kilham, 1988; Wetzel, 2001), less is understood about the seasonal variation in cyanotoxins. The occurrence of cyanobacteria, such as *Aphanizomenon* and *Microcystis*, vary seasonally (Eldridge and Wood, 2019) and one might assume that the toxins they produce vary seasonally as well. However, not all strains of a particular cyanobacterial species are capable of producing toxins (Sipari et al., 2010). It is not known how toxin-producing strains and non-toxin producing strain occurrences differ temporally and the triggers of toxin production are not well understood. Moreover, little is known about how cyanotoxin mixtures vary throughout the open water season and whether peaks in microcystin coincide with peaks in other cyanotoxins.

To help identify when cyanotoxins occur at concentrations that could be dangerous for recreational users, many researchers and water resource managers are turning to computer models that correlate environmental variables to algal biomass or toxin concentrations. Research has shown that concentrations of phosphorus and chlorophyll, Secchi depth, and other environmental factors are related to microcystin occurrence (Kotak et al., 2000). In a study of over 246 water bodies across Canada (Orihel et al., 2012), high microcystin concentrations occurred only at low nitrogen-to-phosphorus ratios in nutrient-rich lakes. In a study of 241 water

bodies in Minnesota, Iowa, Missouri, and Kansas, Secchi depth and latitude were related to microcystin concentrations (Graham et al., 2004). Optical sensors that measure algal or cyanobacterial pigments such as chlorophyll and phycocyanin, have been used to provide early warnings of cyanobacterial presence in recreational waters (Marion et al., 2012) and drinking-water sources (Brient et al., 2008; McQuaid et al., 2011). In a study of lakes in Wisconsin, precipitation, day of the year, stability, and winds were related to the presence of phycocyanin (Stone et al., 2012). The data from these previous studies can be used as a starting point to determine if factors triggering cyanobacterial harmful algal blooms are the same as those triggering the production of cyanotoxins for lakes in Voyageurs National Park.

Some of these parameters have been used by other researchers for input into the U.S. Environmental Protection Agency's Virtual Beach (VB) software (Cyterski et al., 2015). VB was originally designed as a decision support tool for developing site-specific statistical models to predict pathogen indicator (such as *Escherichia coli* and *Enterococci*) concentrations at recreational beaches (Cyterski et al., 2015). Predictive modeling of cyanotoxins is a relatively new application for VB, and has thus far only been used to estimate microcystin concentrations (Francy et al., 2016). Current research is lacking the application of VB for estimating other cyanotoxins, such as saxitoxin and anatoxin-a, or cyanotoxin mixtures.

Another area of this research field that has received little attention, is the biological community and the inter-relationships among cyanobacteria that produce toxins. Gaining insight into how combinations of cyanobacteria are related to toxins and toxin mixtures in recurring blooms, may help increase the understanding of mechanisms that regulate changes in bloom community composition. Therefore, this dissertation examined (1) the spatial and temporal dynamics of the neurotoxins anatoxin-a and saxitoxin and the hepatotoxin microcystin, (2)

related environmental factors that may drive toxin production and their suitability for building a statistical model, and (3) the relations among the bloom community, cyanotoxins, and other secondary metabolites of cyanobacteria. These data will be used by resource managers to determine overall exposure risk to humans, animals, and ecosystems at Kabetogama Lake in Voyageurs National Park.

1.2. Site Description

About 50% of Voyageurs National Park is covered with aquatic habitat, including 30 lakes (Kallemeyn et al., 2003). Some of these lakes suffer from recurring algal and cyanobacterial blooms, providing a unique opportunity to study cyanotoxins. The park is a tourist destination for swimmers, boaters, anglers, and outdoor enthusiasts who may drink water or consume fish from park lakes. Additionally, the lakes support much of the park's fauna, including fish, loons, eagles, beaver, timber wolves, and moose. The effects of cyanotoxins to the broader ecosystem are unknown.

Cyanotoxins are a significant issue in Minnesota where a number of lakes have experienced cyanobacterial blooms that have resulted in human illness and animal deaths (Minnesota Pollution Control Agency, 2013; 2014). For example, cyanobacterial blooms in Kabetogama Lake within Voyageurs National Park frequently produce the toxin microcystin (Christensen and Maki, 2015; Christensen et al., 2011), with some bloom samples exceeding the 1 microgram per liter (μg/L) drinking water guideline of the World Health Organization (WHO; World Health Organization, 2003). Two samples from a previous study (Christensen et al., 2011) were above the WHO high-risk category for recreational exposure (>20 μg/L; Chorus and Bartram, 1999). However, exposure risk estimates are incomplete because no data existed for other cyanotoxins, such as anatoxin-a, saxitoxin, and other secondary metabolites.

Researchers and resource managers have a desire to better understand the timing and triggers for toxin production in cyanobacterial populations. A better understanding of environmental and biological influences on toxin production may lead to more accurate prediction of cyanotoxin occurrence and subsequent prevention and mitigation strategies.

1.3. Research Questions, Objectives, and Hypotheses

The main goal of this dissertation is to increase the understanding of cyanotoxin mixtures, including neurotoxins and other secondary metabolites of cyanobacteria in freshwater environments and to elucidate the value of collecting and analyzing water samples for neurotoxin concentrations, in addition to microcystin concentrations. The drivers of cyanobacterial growth are generally known (Paerl and Otten, 2016), but discerning the drivers of subsequent toxin production is an active area of research (Casali et al., 2017; Rosen et al., 2018). The research on cyanotoxin mixtures, in general, is sparse (Freitas et al., 2014; Graham et al., 2010) and, to date, little is known about combined effects of multiple drivers in terms of overall toxin production. The novelty of this research is that it will be the first known to combine low levels of multiple toxins into a single measure of risk in addition to documenting inter-relations among both cyanobacteria and cyanotoxins in a complex bloom community. The main questions to be addressed by this research are:

1) Do cyanotoxins vary seasonally, with neurotoxin concentrations peaking before microcystin?
2) Are cyanotoxins present prior to visible blooms, putting recreational users at risk?
3) What model (dependent and independent variables) best predicts exposure risk?
4) Is a model with a cyanotoxin mixture better than a model that uses microcystin only?
5) Is the biodiversity of a bloom related to toxin concentrations?

6) Which cyanobacteria or cyanobacterial groups are correlated and how are these related to the toxins and secondary metabolites within the blooms?

Along with these questions, the following objectives were established:

Objective 1. *To determine the seasonal variation in cyanobacteria, toxin-producing potential, and toxin concentrations.* Results include a comparison of cyanobacteria, their toxin-producing ability, and anatoxin-a, microcystin, and saxitoxin concentrations throughout the open water season. It is expected that the microcystin concentrations and the peak in concentration for either anatoxin-a or saxitoxin will not occur at the same time.

Objective 2. *To determine the possible triggers of toxin production through a statistical model and whether a three-cyanotoxin mixture model is more accurate than a model that relies only on microcystin*. Results include a series of models for comparison, using the three toxins (a cyanotoxin mixture) and a single toxin (microcystin) as the dependent variables. Statistical techniques within the U.S. Environmental Protection Agency's (EPA) Virtual Beach (VB) software were used for independent variable selection and to evaluate the predictive capability of each model.

Objective 3. *To determine the co-occurrence between microcystins and other low-polarity toxins as well as determine the similarities among the phytoplankton and cyanobacterial communities in different blooms.* Results include statistical correlations among various cyanobacteria toxins within recurring blooms, as well as indices of similarity between site locations, seasons, and taxa biovolume.

Cyanotoxins are known to cause poisoning in dogs, cats, livestock, wildlife, and humans (Carmichael and Gorham, 1978; Chorus and Bartram, 1999; Vehovszky et al., 2015). Very small exposures, such as a few mouthfuls of cyanotoxin-contaminated water, may result in fatal

poisoning (Trevino-Garrison et al., 2015), and little is known about the effects, either acute or chronic, of cyanotoxin mixtures. These findings amplify the need for understanding exposure to cyanotoxins in northern temperate lakes. The following hypotheses were developed, to help lead to an improved understanding of cyanotoxins exposure:

H1: Summer peak concentrations of anatoxin-a and saxitoxin will occur before peak concentrations of microcystin.

H2: The accuracy of an equation that combines normalized concentrations of multiple toxins for comparison to a health threshold will be greater than the accuracy of an equation that uses a single toxin (microcystin).

H3: Multidimensional analysis will reveal strong relations between cyanobacteria, which may be indicative of synergistic or antagonistic processes.

1.4. Dissertation Organization

This dissertation covers research in one of the large, inter-connected lakes at Voyageurs National Park. Despite the generally pristine lakes and surrounding wilderness, Kabetogama Lake suffers from recurring algal blooms most years that often contain toxin-producing cyanobacteria. Previous work indicated the presence of microcystin (a hepatotoxin) exceeding 100 μg/L (Christensen et al., 2011). However, until this research no analysis on other toxins were completed at the park.

This dissertation has six chapters. This chapter is a general introduction to the topic and covers background, site description, research questions, objectives, and hypotheses, as well as dissertation organization. Chapter 2 is a literature review of the freshwater neurotoxins, primarily anatoxin-a and saxitoxin, for which published information is sparse. The literature described in Chapter 2 is based on the journal article “Freshwater neurotoxins and concerns for human,

animal, and ecosystem health: A review of anatoxin-a and saxitoxin," published in *Science of the Total Environment* (Christensen and Khan, 2020). Chapter 3 presents the first year of sampling at one site and the seasonal variation in phytoplankton, toxin-producing potential, and the toxins microcystin, anatoxin-a, saxitoxin, and cylindrospermopsin. The content of Chapter 3 is derived from the journal article "Phytoplankton community and algal toxicity at a recurring bloom in Sullivan Bay, Kabetogama Lake, Minnesota, USA," published in *Scientific Reports* (Christensen et al., 2019). Chapter 4 is derived from a journal article entitled "Cyanotoxin mixture models: Relating environmental variables and toxin co-occurrence to human exposure risk," published in *Journal of Hazardous Materials* (Christensen et al., 2021b). Chapter 5 is derived from a draft article entitled "Cyanobacterial secondary metabolites and their relation to phytoplankton communities in recurring surface blooms," which will be submitted to a peer reviewed journal. Conclusions and suggestions for future research are included in Chapter 6.

CHAPTER 2: LITERATURE REVIEW[1]

2.1. Introduction

Fossils of cyanobacteria date back 3.5 billion years and are the Earth's oldest oxygen-producing organisms (Schopf, 2002). These microscopic, prokaryotic organisms have had a major effect on the Earth and are responsible for our modern-day oxygen-enriched atmosphere (Paerl and Paul, 2012; Schopf, 2002). Cyanobacteria, therefore, are essential to humans and other organisms that respire aerobically, as well as an important part of the food web, providing food for planktivores and affecting multiple trophic levels.

However, in aquatic environments, excessive reproduction and accumulation of cyanobacteria can lead to the formation of cyanobacterial blooms (Orihel et al., 2015; Zhao et al., 2019). These blooms can cause water supply and treatment issues, restrict recreation (Calado et al., 2019), deplete dissolved oxygen (Janssen, 2019; Paerl, 1988), and result in fish mortality (Sabart et al., 2015). More importantly, cyanobacteria that produce toxic metabolites, called cyanotoxins, are a global concern (Chorus and Bartram, 1999) because they may adversely affect humans, animals, and ecosystems.

Cyanotoxins are classified into three main groups based on their target tissue: dermatoxins, hepatotoxins, and neurotoxins (Chorus and Bartram, 1999). The most frequently studied freshwater toxin is the hepatotoxin microcystin (Merel et al., 2013) and numerous reviews of microcystin have been published (e.g., Díez-Quijada et al., 2019; Massey et al., 2018; Preece et al., 2017; Welten et al., 2019). However, neurotoxins have different modalities and

[1] The material in this chapter was co-authored by Victoria Christensen and Dr. Eakalak Khan. Victoria Christensen had primary responsibility for the literature research, writing the draft, and all revisions of this chapter. Victoria Christensen is the primary developer of the conclusions and research gaps that are advanced here, and Dr. Eakalak Khan proofread and served as advisor.

modes of action than microcystin and, therefore, likely behave differently on organisms and in the environment.

Neurotoxin groups include anatoxins, saxitoxins, ciguatoxins, and beta-N-methylamino-L-alanine (BMAA; Rutkowska et al., 2019). Three of these groups are known to be produced in freshwater environments: anatoxins, saxitoxins, and BMAA. Anatoxin-a and saxitoxin are two freshwater neurotoxins that have been linked to acute animal poisonings and, therefore, may have unknown ecological effects in wildlife and in ecosystems. Anatoxin-a has been implicated in animal mortality and can cause death in minutes (Carmichael and Boyer, 2016; Edwards et al., 1992; Heiskary et al., 2014; Sabart et al., 2015; Wood et al., 2007). Saxitoxin, which is well-studied in marine environments, is one of the most potent naturally occurring neurotoxins known (Cusick and Sayler, 2013; Loftin et al., 2016; Wiese et al., 2010). Despite the potency of these neurotoxins, anatoxin-a and saxitoxin are understudied in freshwater environments. Therefore, the focus of this critical review is on the neurotoxins anatoxin-a and saxitoxin and their effects on humans, animals, and freshwater ecosystems. This review provides background information on anatoxin-a and saxitoxin, in addition to (1) tables of research identifying cyanobacterial species with confirmed production of anatoxin-a and saxitoxin in isolated organisms, and (2) tables of research identifying locations and concentrations anatoxin-a occurrence in the environment for other researchers to utilize in their work on cyanotoxins in freshwater systems.

2.1.1. Connection Between Cyanobacteria and Cyanotoxins

Some cyanobacteria have ecological niches that help them dominate over other cyanobacteria. Water clarity, total phosphorus, nitrogen, macrophyte cover, dissolved oxygen levels, water depth, and chemical oxygen demand can all play a role in cyanobacterial community composition (Beaver et al., 2018; Dalu and Wasserman, 2018), and the response

these environmental conditions are likely taxon specific. For example, *Aphanizomenon* can outcompete *Anabaena* in light-limited conditions (De Nobel et al., 1998). Furthermore, *Anabaena* (Wood et al., 2010b) and *Aphanizomenon* (Moustaka-Gouni et al., 2017) can fix nitrogen from the atmosphere, which may play a role in their dominance in nitrogen-poor lakes. Nitrogen fixation is made possible by the formation of heterocytes, whereas other specialized cells, called akinetes, allow the cyanobacteria to remain dormant in sediments and survive harsh or even extreme conditions, reviving when conditions for growth are right (Moustaka-Gouni et al., 2017). The formation of heterocytes and akinetes may be why certain cyanobacteria and their toxins are so successful at surviving and persisting in the environment (Kaplan-Levy et al., 2010).

Salinity treatments were shown to reduce cyanobacterial cell membrane integrity (Rosen et al., 2018), and Li et al. (2015) determined that cyanobacterial blooms occurred more frequently at salinities below 5 practical salinity units. Conversely, some cyanobacteria, such as *Aphanizomenon favaloroi,* can withstand salt stress, giving them an advantage in brackish waters (Moustaka-Gouni et al., 2017). Whereas optimal conditions for some taxa have been characterized, the conditions that lead to dominance of one organism over another are complex. Moreover, the conditions that lead to the presence of toxin-producing strains within each species of cyanobacteria are not well understood.

Cyanotoxin production by cyanobacteria is believed to be an ancient trait. Saxitoxin, for example, was present 2.1 billion years ago (Murray et al., 2011). As such, the cyanotoxins target fundamental cellular processes in a wide range of organisms. Cyanotoxins may have originated as a defense mechanism against grazing pressure or competition; some cyanotoxins are allelopathic, inhibiting the growth of other organisms such as algae that compete for resources

(Christoffersen, 1996; Holland and Kinnear, 2013). Another plausible explanation is that cyanotoxins contribute to cellular physiology by improving homeostasis, photosynthesis, or growth rates (Holland and Kinnear, 2013). Alternatively, the production of toxins may be a mechanism that shapes the cyanobacterial community as a whole rather than individual organisms, the differing niches and traits among individuals contributing to the survival of cyanobacteria (Wang et al., 2020). Janssen (2019) raised the question of whether some cyanotoxins have no ecotoxicological significance or if they have received too little scientific attention to determine their ecological function.

Cellular cyanotoxin content is specific to the cyanobacterial strain (Chorus and Bartram, 1999) and may vary by up to four orders of magnitude (Christoffersen, 1996). Therefore, low abundances of some organisms may still result in high cyanotoxin concentrations, and understanding cyanobacterial accumulations are important. Under certain conditions, such as warmer temperatures, adequate light, low manganese, or high nutrient inputs (Feuchtmayr et al., 2010; Orihel et al., 2015), cyanobacterial abundance increases rapidly. Excess nutrient inputs, in the form of nitrogen and phosphorus from both natural and human sources, have received attention as a primary cause of cyanobacterial blooms (Agnihotri, 2014; Wang et al., 2010), although cyanotoxin presence in oligotrophic lakes is causing some to challenge the current paradigms (Carey et al., 2012; Glibert, 2017; Reynolds, 1998). The decoupling of cyanotoxins, either spatially or temporally, from cyanobacteria is a concern due to the lack of visual cues that cyanotoxins are present (Christensen et al., 2019).

2.1.2. Previous Cyanotoxin Reviews

Several researchers have reviewed the literature on cyanobacteria (e.g. Ger et al., 2014; Quiblier et al., 2013), but reviews on cyanotoxins other than microcystin, and to a lesser extent

cylindrospermopsin, are sparse. Most cyanotoxin papers focus on certain aspects of cyanotoxins. For example, Preece et al. (2017) covered the occurrence of cyanotoxins in coastal environments, and Moy et al. (2016) reported on the biotransport of cyanotoxins to riparian food webs. Other aspects of cyanotoxins covered in the literature include the effects of sample preparation and storage on cyanotoxin analysis (Kamp et al., 2016), effects of exposure, including acute animal and human poisonings and fatalities (Carmichael et al., 2001; Wood, 2016), exposure routes (Codd et al., 1999; Facciponte et al., 2018), exposures to toxins in health supplements (Dietrich et al., 2008), bioaccumulation (Al-Sammak et al., 2014), or negative and positive aspects of cyanobacteria and cyanotoxins, ranging from cancer-causing to cancer-fighting properties (Zanchett and Oliveira-Filho, 2013). At least one researcher reviewed extreme environments (Cirés et al., 2017), concluding that cyanotoxins can thrive in hot springs, polar deserts, alkaline lakes, and hypersaline environments.

However, with the exception of a few papers (e.g. Aráoz et al., 2010; D'Anglada et al., 2015; Osswald et al., 2007; Rutkowska et al., 2019), most papers are not specific to neurotoxins and, none specifically focused on freshwater neurotoxins and their effects on animals and ecosystems. Janssen (2019) went beyond microcystin and other low molecular weight toxins and reviewed products of cyanobacteria called cyanobacterial peptides, reporting cyanobacterial peptides can occur just as frequently and at similar concentrations as microcystins. Building from Janssen's (2019) question of whether some cyanobacterial metabolites have no ecotoxicological significance or whether they simply have received too little attention, this review extends this question to the understudied neurotoxins, anatoxin-a and saxitoxin.

2.2. The Neurotoxins—Categories and Mode of Action

Neurotoxins are a group of compounds that have clear biological effects on the nervous system but differ in chemical structure and mode of action (Rutkowska et al., 2019). Anatoxins and saxitoxins are neurotoxin classes with numerous variants. The anatoxins consist of three categories: anatoxin-a, homoanatoxin-a, and anatoxin-a(s). However, anatoxin-a(s) is structurally unrelated to anatoxin-a and homoanatoxin-a (Miller et al., 2017; Rutkowska et al., 2019), and a recent suggestion to rename it guanitoxin was proposed (Fiore et al., 2020). Saxitoxin has more than 50 analogues (Miller et al., 2017; Wiese et al., 2010) including nonsulphated (saxitoxin and neosaxitoxin), monosulphated (gonyautoxins), disulphated (C-toxins), and decarbamoyl variants and derivatives (Chorus and Bartram, 1999; Wiese et al., 2010). Another neurotoxin produced by cyanobacteria, beta-N-methylamino-L-alanine (BMAA), typically is associated with soil but also can be produced by freshwater cyanobacteria (Cox et al., 2005; Jiao et al., 2014; Metcalf et al., 2008). The neurological effects of BMAA have been debated, primarily their reported connection with neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) and Parkinson's disease (Chernoff et al., 2017). Homoanatoxin-a, anatoxin-a(s), and BMAA (and its isomers), will not be covered in detail here, but their classification relative to other freshwater cyanotoxins/neurotoxins is shown (Fig. 2.1).

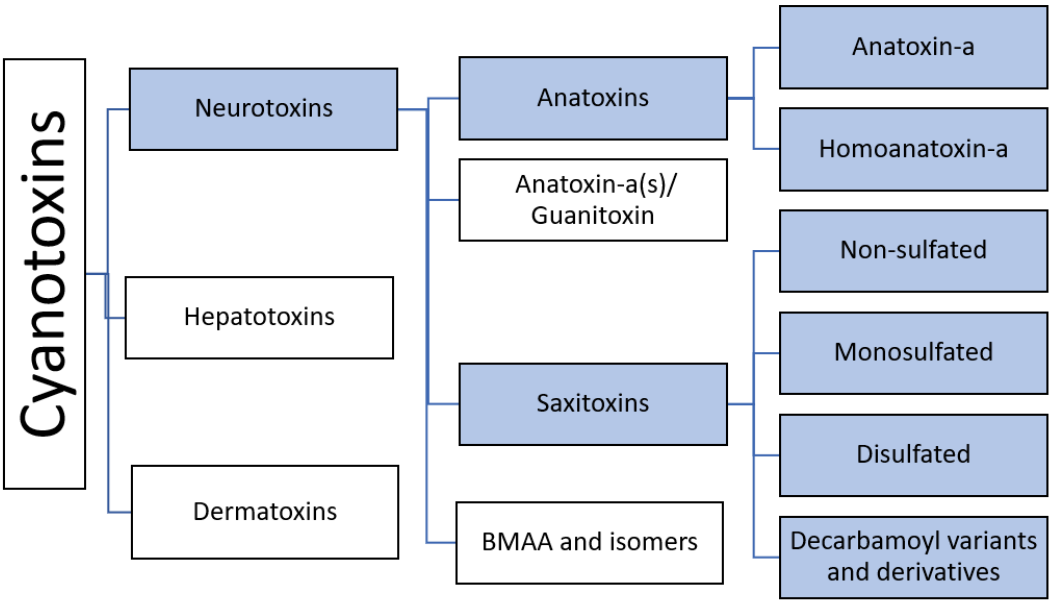

Figure 2.1. Neurotoxin classes based on mode of action (after Aráoz et al., 2005; Mello et al., 2018; Rutkowska et al., 2019).

Anatoxins and saxitoxins are both neurotoxic alkaloids (Humpage et al., 2005; Rutkowska et al., 2019). Alkaloids are naturally occurring organic compounds that contain nitrogen (Robinson, 2016), such as morphine, strychnine, and nicotine—all of which have major physiological effects. In the case of the anatoxins and saxitoxins, the physiological effects are on the nervous system, interfering with nerve cells throughout the body and the messages these nerve cells send to the brain.

2.2.1. Anatoxins—Mode of Action

Anatoxin-a (molecular weight, MW=165; Chorus and Bartram, 1999) and homoanatoxin-a (MW=179; Chorus and Bartram, 1999) mimic acetylcholine (a neurotransmitter, similar to dopamine or adrenaline) and bind to acetylcholine receptors at the synapses between nerves and muscle tissue. However, anatoxins are not degraded by acetylcholinesterase (D'Anglada et al., 2016), thus muscles become over-stimulated, leading to fatigue (Kotak and Zurawell, 2007). The

potency of homoanatoxin-a is potentially greater than anatoxin-a because of increases in acetylcholine release into neuromuscular synapses (Rutkowska et al., 2019). The chemical structure of anatoxin-a has been described as most closely related to cocaine (Carmichael and Gorham, 1978), and in fact anatoxin-a has been synthesized through ring extraction of cocaine (Carmichael et al., 1985). Because the over-stimulated muscles include those involved in respiration, anatoxin-a poisoning can cause death by respiratory failure (Al-Sammak et al., 2014).

Anatoxin-a(s) (MW=252; Patočka et al., 2011) is an organophosphate that has been described as similar to an organophosphorus or carbamate insecticide (Metcalf and Bruno, 2017). The letter "s" stands for "salivation," one of the characteristic symptoms of anatoxin-a(s) poisoning (Patočka et al., 2011). Other symptoms may include urinary incontinence, lacrimation, convulsions, and respiratory distress (Mahmood and Carmichael, 1986). Anatoxin-a(s) inhibits acetylcholinesterase (Matsunaga et al., 1989). The result is that acetylcholine is not hydrolyzed at the synapse, blocking the nerve influx (Metcalf and Bruno, 2017). Consequently, acetylcholine is available to bind membrane receptors, leading to continuous muscle simulation and potential respiratory failure and brain hypoxia (Patočka et al., 2011).

2.2.2. Saxitoxins—Mode of Action

Saxitoxins block sodium channels along nerve cells, which then suppress the transmission of a nerve impulse (Kotak and Zurawell, 2007; O'Neill et al., 2016). Subsequently, the stimulation of muscles is suppressed, including those associated with breathing (Chorus and Bartram, 1999), resulting in respiratory paralysis. Whether under-stimulation of muscles due to saxitoxin, or over-stimulation of muscles due to anatoxin-a, the result of a lethal dose is essentially the same: death by respiratory failure.

Saxitoxin (MW=299), is the most researched paralytic shellfish toxin (PST), and its analogues have varying levels of toxicity, with saxitoxin and neosaxitoxin being the most potent (Ballot et al., 2010; Wiese et al., 2010). The most common PSTs are hydrophilic, although hydrophobic saxitoxins have been found exclusively in freshwater environments (Wiese et al., 2010). The introduction of a hydrophobic side chain in the structure of some saxitoxin analogues results in a decrease in the binding to receptors on sodium channels and thus lower toxicity (Onodera et al., 1997a). Saxitoxin is lipophilic, indicating the toxin sorbs onto fats and could be a concern for bioaccumulation (Negri and Jones, 1995; Wiese et al., 2010). In some cases, saxitoxin toxicity is expressed in terms of saxitoxin equivalents, calculated by using results obtained with a saxitoxin standard, due to differences of the animal used in the bioassay (e.g. strain, sex, and condition; Suzuki and Machii, 2014).

The toxicity of anatoxin-a and saxitoxin is compared to other toxins (Fig. 2.2). It is evident that saxitoxin is potentially the most potent freshwater toxin and warrants special consideration. However, this figure and most toxicity data available are based on intraperitoneal (i.p.) injection of toxins. Whereas i.p. injections in mice are an efficient method of determining the lethal dose required to cause mortality in 50% of subjects (LD50) and provide information on comparative toxicity, humans and other mammals are likely to be exposed through the oral pathway. Most of the focus on saxitoxin has been in marine environments where the pathway of interest is through the consumption of shellfish. Studies on oral exposure are less common but would be especially relevant for saxitoxin in freshwater.

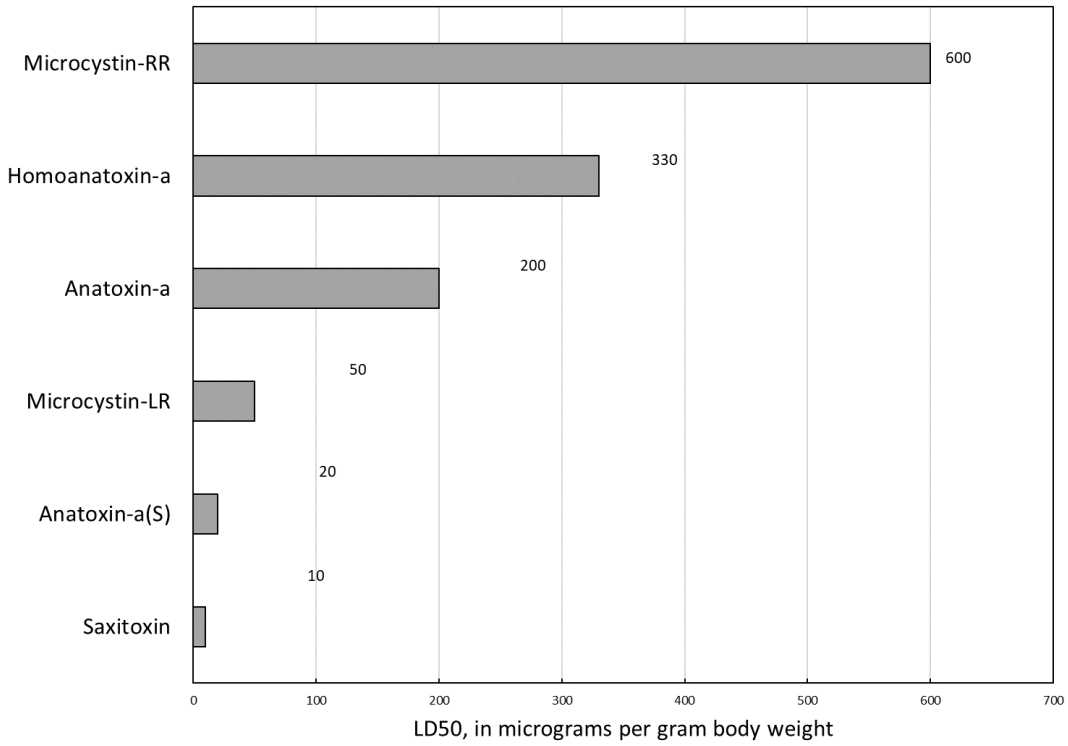

Figure 2.2. Acute toxicity (LD50) of common cyanotoxins, based on intraperitoneal mouse bioassay. [LD50, the dose that would cause mortality in 50 percent of a population; data from: Ballot et al., 2017; Carmichael et al., 1990; Carmichael and Boyer, 2016; Chorus and Bartram, 1999; Dittmann and Wiegand, 2006.]

2.3. Anatoxin-a and Saxitoxin—Production, Isolation, and Identification

The earliest cases of anatoxin-a and saxitoxin in freshwater environments were reported in the 1960s. Anatoxin-a, isolated from a cyanobacterial accumulation that killed cattle, was first called Very Fast Death Factor because it killed mice in 2–5 minutes (Devlin et al., 1977; Gorham et al., 1964). The earliest freshwater detection of another potent toxin was isolated from a strain of *Aphanizomenon flos-aquae* from Kezar Lake in New Hampshire, USA (Sawyer et al., 1968). This potent toxin was later called saxitoxin (Carmichael et al., 1985). Saxitoxin was named after *Saxidomus gigantus*, the first shellfish in which it was identified. Saxitoxin has been found in both marine and freshwater environments, yet saxitoxin in marine environments has received more attention as one of several PSTs (Rutkowska et al., 2019) known for their role in acute

paralytic shellfish poisoning (O'Neill et al., 2016). However, saxitoxin also has been detected in freshwater mussels (Negri and Jones, 1995) and snails (Qiao et al., 2018), leading to the potential for human and animal exposure in freshwater systems. Although saxitoxin has been detected in both marine and freshwater environments (Rutkowska et al., 2019), anatoxin-a is only known to be produced by freshwater cyanobacteria (Kotak and Zurawell, 2007).

2.3.1. Anatoxin-a and Saxitoxin Producers

During the course of a growing season, cyanobacterial communities may shift from toxic to non-toxic strains of the same species (Bozarth et al., 2010; Christensen et al., 2019), and strain composition may be diverse. One difficulty in identifying toxin-producing cyanobacteria species is that there can be intraspecies variation in the genetic capacity to produce toxins, which may be a result of the balance between the cost of toxin production versus resource uptake and growth (Matthews et al., 2020). Toxic and non-toxic strains cannot be differentiated by microscopy, but can be differentiated by molecular detection methods, such as quantitative polymerase chain reaction (qPCR). Considering only the papers with confirmed production in isolated organisms, numerous cyanobacteria species were identified as producing anatoxin-a (Table 2.1) and saxitoxin (Table 2.2). Based on experimental evidence, over 40 freshwater species can produce anatoxin-a compared to 15 species that produce saxitoxin. This may indicate that fewer species produce saxitoxin in freshwater environments than anatoxin-a or that the current data are biased toward anatoxin-a producing species.

Table 2.1. Cyanobacteria species identified as producing anatoxin-a, based on isolated strains. Generally, species are referred to here as they are in each paper cited. See footnotes for details.

Cyanobacteria species	Reference
Anabaena sp. [1]	Sivonen et al. (1989); Park et al. (1993); Harada et al. (1993); James et al. (1997); Rapala and Sivonen (1998); Bumke-Vogt et al. (1999); Ghassempour et al. (2005); Trainer and Hardy (2015)
Anabaena circinalis	Sivonen et al. (1989); Harada et al. (1993); Bumke-Vogt et al. (1999)
Anabaena crassa	Bumke-Vogt et al. (1999)
Anabaena flos-aquae	Carmichael et al. (1975); Devlin et al. (1977); Carmichael and Gorham (1978); Sivonen et al. (1989); Harada et al. (1993); Kangatharalingam and Priscu (1993); Rapala et al. (1993); Gallon et al. (1994); Bumke-Vogt et al. (1999); Qian et al. (2017)
Anabaena lemmermannii	Onodera et al. (1997)
Anabaena macrospora	Park et al. (1993)
Anabaena mendotae	Rapala et al. (1993)
Anabaena planctonica	Bruno et al. (1994); Bumke-Vogt et al. (1999)
Anabaena spiroides	Park et al. (1993)
Aphanizomenon sp.	Sivonen et al. (1989); Harada et al. (1993); Bumke-Vogt et al. (1999); Trainer and Hardy (2015)
Aphanizomenon gracile	Ballot et al. (2010); Cirés et al. (2017); Savela et al. (2017)
Aphanizomenon favaloroi	Moustaka-Gouni et al. (2017)
Aphanizomenon. flos-aquae	Jackim and Gentile (1968); Sawyer et al. (1968); Alam et al. (1973); Ikawa et al. (1982); Pereira et al. (2000); Ferreira et al. (2001)
Aphanizomenon issatchenkoi	Li et al. (2003)
Aphanotece	Bumke-Vogt et al. (1999)
Arthospira fusiformis	Ballot et al. (2004); Ballot et al. (2005)
Cylindrospermopsis	Trainer and Hardy (2015)
Cylindrospermopsis raciborskii	Bumke-Vogt et al. (1999)
Cylindrospermum	Sivonen et al. (1989); Trainer and Hardy (2015)
Gomphosphaeria	Bumke-Vogt et al. (1999)
Limnothrix	Bumke-Vogt et al. (1999)
Lyngbya sp.	Bumke-Vogt et al. (1999)
Microcystis	Park et al. (1993); Bumke-Vogt et al. (1999)
Microcystis aeruginosa	Harada et al. (1993)
Nostoc carneum	Ghassempour et al. (2005)
Oscillatoria	Sivonen et al. (1989); Edwards et al. (1992); Harada et al. (1993); James et al. (1997); Bumke-Vogt et al. (1999); Hamill (2001); Aráoz et al. (2005); Cadel-Six et al. (2007); Trainer and Hardy (2015)
Oscillatoria agardhii	Sivonen et al. (1989)
Oscillatoria formosa	Aráoz et al. (2005)
Oscillatoria limnetica	Osswald et al. (2009)
Phormidium	Cadel-Six et al. (2007); Faassen et al. (2012); Wood et al. (2012)
Phormidium favosum	Gugger et al. (2005)
Phormidium autumnale	Wood et al. (2007); Wood et al. (2010)

Table 2.1. Cyanobacteria species identified as producing anatoxin-a, based on isolated strains (continued). Generally, species are referred to here as they are in each paper cited. See footnotes for details.

Cyanobacteria species	Reference
Phormidium cf. *uncinatum*	Harland et al. (2014)
Planktothrix	Trainer and Hardy (2015)
Planktothrix favosum	Gugger et al. (2005)
Planktothrix rubescens	Viaggiu et al. (2004)
Planktolyngbia	Bumke-Vogt et al. (1999)
Synechocystis	Bumke-Vogt et al. (1999)
Pseudanabaena	Bumke-Vogt et al. (1999)
Raphidiopsis	Trainer and Hardy (2015)
Raphidiopsis mediterranea	Namikoshi et al. (2003)
Tychonema bourrellyi	Shams et al. (2015)

[1]The planktonic *Anabaena* sp. was later classified as *Dolichospermum* (Wacklin et al., 2009; Li et al., 2016)

Most studies identified *Dolichospermum* (*Anabaena*) and *Aphanizomenon* as the cyanobacteria species that produce anatoxin-a (Chernova et al., 2017). Nomenclature, however, is in constant flux and in fact, Komarek and Anagnostidis (1989) estimated that 50% of cyanobacteria in culture collections are misidentified. Some researchers have reported that the reclassification of some cyanobacteria species has only added to the confusion over toxin production. For example, the pelagic form of *Anabaena* has been reclassified as *Dolichospermum* (Wacklin et al., 2009; Li et al., 2016) and therefore in this paper, occurrences of this genera are referred to as *Dolichospermum*, except in the tables where the species is identified according to the authors original classification because agreement over the change in nomenclature is not universal among researchers. In another example, Pereira et al. (2004b) reported that some *Aphanizomenon flos-aquae* have been reclassified as *Aphanizomenon* sp. after careful examination or ribosomal RNA (rRNA) gene sequencing analysis. This careful re-examination indicates that identifying cyanobacteria species by morphological characteristics alone is a challenge and is perhaps part of the reason confusion exists over toxin production.

Table 2.2. Cyanobacteria species identified as producing saxitoxin, based on isolated strains. Generally, species are referred to here as they are in each paper cited. See footnotes for details.

Cyanobacteria species	Reference
Anabaena[1]	Trainer and Hardy (2015)
Anabaena circinalis	Negri and Jones (1995); Negri et al. (1997); Jones and Negri (1997)
Aphanizomenon sp.	Trainer and Hardy (2015)
Aphanizomenon gracile	Pereira et al. (2004b); Casero et al. (2014); Savela et al. (2017)
Aphanizomenon favaloroi	Moustaka-Gouni et al. (2017)
Aphanizomenon flos-aquae	Jackim and Gentile (1968); Sawyer et al. (1968); Alam et al. (1973); Ikawa et al. (1982); Pereira et al. (2000); Ferreira et al. (2001)
Aphanizomenon issatschenkoi	Nogueira et al. (2004)
Cylindrospermopsis raciborskii	Lagos et al. (1999); Castro et al. (2004); Lopes et al. (2017); Mesquita et al. (2019)
Cylindrospermopsis stagnale	Borges et al. (2015)
Lyngbya wollei	Carmichael (1997); Onodera et al. (1997b); Yin et al. (1997); Foss et al. (2012); Lajeunesse et al. (2012); Smith et al. (2019)[2]
Phormidium	Borges et al. (2015)
Planktothrix	Pomati et al. (2000); Trainer and Hardy (2015)
Raphidiopsis brookii	Yunes et al. (2009)
Scytonema cf. *crispum*	Smith et al. (2011); Smith et al. (2012); Belykh et al. (2016)
Woronichinia	Harland et al. (2015)

[1]The planktonic *Anabaena* sp. was later classified as *Dolichospermum* (Wacklin et al., 2009; Li et al., 2016;).
[2]*Lyngbya wollei* is referred to as *Microseira* (*Lyngbya*) *wollei* by Smith et al., (2019).

Adding to the challenge of identifying toxin producers is that numerous strains of cyanobacteria exist. A study determined that in cyanobacteria strains isolated from Japanese lakes, one strain of *Anabaena planctonica* produced anatoxin-a, whereas a second strain did not (Park et al., 1993). Another study determined that strains of *Anabaena* in North America produced anatoxin-a, whereas similar strains of the same species in Australia produced saxitoxin, but not anatoxin-a (Negri et al., 1997), although anatoxin-a has been recently detected in Australia (John et al., 2019). Several different strains of *Anabaena* and *Aphanizomenon* have been identified as anatoxin-a and saxitoxin producers in freshwater habitats (Codd et al., 1999), yet in laboratory tests of 92 strains of *Anabaena*, *Aphanizomenon*, and *Anabaenopsis* from German lakes, Ballot et al. (2010) identified 14 strains of *Aphanizomenon gracile* that produced four saxitoxin variants, whereas none of the 92 strains produced anatoxin-a.

In a study of Lake Crato in Portugal (Pereira et al., 2000), saxitoxin was produced by an accumulation of *Aphanizomenon flos-aquae*. This cyanobacteria species was present early in the season, later being taken over by *Microcystis aeruginosa*, a microcystin producer. In another Portugal water body, Ferreira et al. (2001) also showed that *Aphanizomenon flos-aquae* gradually was replaced by *Microcystis aeruginosa* later in the season. These studies are indications that a natural progression of species throughout a season may be the norm and that different toxins may be produced at different times of year, but may also co-exist, throughout a cyanobacterial bloom.

2.3.2. Factors Leading to Toxin Production

Temperature, light, salinity, and nutrient conditions have been shown to affect production of cyanotoxins (Merel et al., 2013) and neurotoxins (Rapala et al., 1993) by cyanobacteria cells. High temperature and growth-limiting low light decreased anatoxin-a production by *Anabaena* and *Aphanizomenon*, whereas growth-limiting high light decreased anatoxin-a production by *Anabaena* but not *Aphanizomenon* (Rapala et al., 1993). Salinity can also be a factor in toxin production, and recent research showed that microcystin production is greatest in low salinity environments (<18 practical salinity units; Rosen et al., 2018). Neurotoxins may have similar salinity preferences, although little research was found that identified salinity in anatoxin-a or saxitoxin production in freshwater environments. One notable study (Pomati et al., 2004) demonstrated that 10 millimole (mM) NaCl inhibited *Cylindrospermopsis raciborskii* growth while promoting intracellular saxitoxin accumulation at doses of 1, 5, and 10 mM.

Nitrogen and phosphorus have been linked to cyanobacterial growth, but the link between these nutrients and toxin production is unclear. In a study of a lake in China (Qian et al., 2017), anatoxin-a concentrations varied depending on the nitrogen source, with nitrogen from urea resulting in the highest concentrations. Rapala et al. (1993) showed that *Anabaena* and

Aphanizomenon produced more anatoxin-a when grown in a nitrogen-free medium (requiring nitrogen fixation) than a nitrogen-rich medium, but orthophosphate concentrations had no effect on anatoxin-a concentrations. When looking into the drivers of saxitoxin production, Casali et al. (2017) reported that production of saxitoxin in *Cylindrospermopsis raciborskii* was both nutrient and cell density dependent, with the highest toxin production at the highest nutrient (nitrate and orthophosphate) concentrations but at the lowest cell densities, which the authors interpreted as a stress adaptation of saxitoxin-producing strains.

Toxin production also could be triggered by environmental conditions, such as water movement or residence time (Merel et al., 2013), high water temperature (e.g. Casero et al., 2014; Yin et al., 1997), or a combination of factors. The relative quantities of carbon and nutrients has been suggested as a trigger for microcystin production (Van De Waal et al., 2009), and stoichiometry could play a role in neurotoxin production as well. However, the optimal environmental preferences for production remain uncertain for most cyanotoxins and in some cases environmental conditions may influence cyanobacterial strain composition rather than cyanotoxin production.

2.4. Environmental Fate

Once anatoxin-a and saxitoxin are produced, their persistence in the aquatic environment will depend, to some extent, on persistence traits in the cyanobacteria species that produce them. For example, the ability of *Aphanizomenon* to fix nitrogen allows it to remain in low nitrogen environments (Moustaka-Gouni et al., 2017; Wood et al., 2010b), and the salt tolerance of *Aphanizomenon favaroloi* can help it thrive in both saline environments (Moustaka-Gouni et al., 2017) and in sediments as dormant, environmentally resistant akinetes (Kaplan-Levy et al., 2010). Additionally, physical factors in the environment (e.g. temperature, ultraviolet radiation)

can contribute to cyanobacteria and toxin accumulation in blooms (Kaminski et al., 2013). However, production, persistence, and degradation all contribute to the toxins present in freshwater environments.

2.4.1. Intracellular and Extracellular Toxins

After cyanotoxins are produced, they may either remain in the cyanobacterial cell (intracellular) or be released into the water (extracellular) when cells die and rupture (Lopes et al., 2017; Merel et al., 2013). Toxins are released into the freshwater environment almost exclusively during cell senescence, death, and lysis, and toxin release does not appear to occur continuously, except in the case of anatoxin-a, which may leak out of cells during the growth phase in low light environments (Chorus and Bartram, 1999). The cause of cell senescence and lysis is the focus of recent research, primarily for the cyanobacterial species that produce microcystin (Kramer et al., 2018; Rosen et al., 2018; Walls et al., 2018a).

In one study of 80 German lakes (Bumke-Vogt et al., 1999), anatoxin-a was detected more often in cells (intracellular) than in water (extracellular), but extracellular concentrations were higher. Although extracellular toxins released into the water column can be high when a cyanobacterial bloom ages (Jones and Orr, 1994), concentrations are usually not sustained due to dilution, wind mixing, adsorption, and biodegradation (Funari and Testai, 2008). For example, toxins released from cells in lakes and rivers are diluted by large amounts of water, especially in areas where strong winds or currents mix the water rapidly (Jones and Orr, 1994). In terms of poisoning risk, however, Funari and Testai (2008) argue that it is the combination of intra- and extracellular toxin concentrations that is important.

2.4.2. Toxin Persistence, Degradation, and Half-life

Microcystins retained inside cyanobacterial cells may persist for months (Chorus and Bartram, 1999). Less is known about the intracellular persistence of neurotoxins, but once released from the cell, the persistence of cyanotoxins in the environment depends on the structure of the toxin (Klitzke et al., 2011), local environmental conditions including endemic bacterial populations (Jones and Orr, 1994), and the efficiency of the degradation process, which can include hydrolysis, photolysis, and bacterial degradation (Funari and Testai, 2008).

2.4.2.1. Anatoxin-a — The Effects of pH, Sunlight, Temperature, and Sediment

Anatoxin-a is water soluble (Merel et al., 2013) and stable in acidified (pH <3) conditions (Kaminski et al., 2013), but degrades in alkaline conditions (D'Anglada et al., 2016; Kaminski et al., 2013; Merel et al., 2013; Stevens and Krieger, 1991a). The half-life of anatoxin-a was estimated as 1–2 hours under expected light and pH conditions (pH 8-9) of a decaying bloom in most northern temperate climates (Stevens and Krieger, 1991a). Anatoxin-a undergoes rapid degradation to non-toxic forms in sunlight (Kaminski et al., 2013; Osswald et al., 2007; Stevens and Krieger, 1991a), a common condition in the late summer months when blooms and toxins are most likely. However, half-life increased to about 5 days in the absence of light (at pH 9; (Stevens and Krieger, 1991b). It is possible that extensive floating algal mats could block sunlight to such a degree that anatoxin-a would thrive in the water column for longer periods of time.

In addition to pH and sunlight, Kaminski et al. (2013) reported other physiochemical factors that may be related to anatoxin-a fate in many environments, including high photosynthetically active radiation (PAR). Visible light, or PAR, is the wavelength important in aquatic primary production (Kallemeyn et al., 2003). At high PAR (at 9.5 pH) in the absence of

other light forms, Kaminski et al. (2013) recorded only a slight degradation of anatoxin-a, whereas ultraviolet-B (UVB) radiation (at pH 7) reduced anatoxin-a by 82% in 1 hour. However, very little of the light that reaches the Earth's surface is UVB radiation—over 90% of all ultraviolet light is blocked by the ozone layer (Ben-Yakir and Fereres, 2016). Therefore, substantial degradation by UVB is not likely to happen under natural conditions.

Kaminski et al. (2013) reported that low temperatures (below 20 ºC) can lead to anatoxin-a persistence, and high temperatures and neutral or high pH can lead to anatoxin-a degrading more rapidly. However, Kaminski et al. (2013) emphasized that high temperature is not the only factor in anatoxin-a degradation; other physiochemical factors must be considered because both production and degradation contribute to the amount of anatoxin-a in water.

The variable nature of anatoxin-a degradation may be a consequence of different organisms that degrade it (Rapala et al., 1994), and many of these organisms are found within the sediment. Cells that settle into bottom sediment may undergo rapid breakdown by bacteria and protozoa (Chorus and Bartram, 1999). The half-life of anatoxin-a in sediment has been reported as about 5–10 days (Rapala et al., 1994). Sorption to sediment also is an important pathway for anatoxin-a elimination from the water column, primarily through cation exchange, with strong sorption to organic rich clays and muds and weak sorption in sandy soils (Klitzke et al., 2011). However, the sorption between layers of clay may decrease the availability of anatoxin-a to microbes, which may lead to slower degradation and the toxin may remain ecologically available for a substantial amount of time (Bouaïcha and Corbel, 2016). Therefore, sediment texture and chemical structure are important considerations for determining the fate of a toxin in sediment (Klitzke et al., 2011), which may include breakdown of the toxin cells and re-release into the overlying water column (Chorus and Bartram, 1999).

2.4.2.2. Saxitoxin —The Effects of pH, Temperature, and Sediment

Like anatoxin-a, saxitoxin is water soluble (Kamp et al., 2016; Trainer and Hardy, 2015), and the stability of saxitoxin is thought to depend on pH (Castro et al., 2004; Pereira et al., 2004a). In an early laboratory study of *Aphanizomenon flos-aquae* (Jackim and Gentile, 1968), a saxitoxin-like toxin was produced, which was stable at a pH of 2–4 but became less stable with increasing pH. Pereira et al. (2004a) confirmed this with an experiment where total PSTs remained stable at a pH of 3 but decreased exponentially at pH 7 and 9. However, unlike anatoxin-a, saxitoxins and other PSTs often transform from low toxicity C-toxins to high toxicity dicarbamoyl-gonyautoxins, resulting in a short-term increase in sample toxicity, by as much as 6 times after 10 days (Jones and Negri, 1997; Pereira et al., 2004a). This transformation to higher toxicity was further demonstrated in a study where extracellular saxitoxin concentrations increased consistently for 1–3 weeks (Harland et al., 2015).

The half-life of saxitoxin is pH and temperature dependent. For saxitoxin and its analogues at 25 ºC and neutral pH, half-life ranged from about 9 to 28 days in irrigation drain water and river water, and up to 69 days in sterile water (Jones and Negri, 1997). In one laboratory test, the saxitoxin analogue gonyautoxin persisted for over 90 days in a freshwater experiment, with 30% of the initial concentration remaining at the end of the experiment (Jones and Negri, 1997). Half-lives increased two- to three-fold, depending on the analogue, when temperature was decreased from 30 ºC to 20 ºC (at neutral pH; Pereira et al., 2004a). Therefore, saxitoxins are very stable at biologically relevant pH and temperatures (Harland et al., 2015), particularly for freshwater systems during summer months.

Burns et al. (2009) reported that saxitoxin cells can settle out of the water column, adsorb to clays through cation exchange, and possibly persist for years. Although salt was introduced to

their freshwater experiments, Burns et al. (2009) indicated the potential of sediments to preserve saxitoxin cells. In a water treatment experiment (Kayal et al., 2008), organisms in anthracite from filter beds biotransformed saxitoxin to more toxic analogues.

No studies were found that examined saxitoxin breakdown under sunlight. Few studies were found that examined the degradation of saxitoxins in freshwater environments in general, but studies showed saxitoxin breaks down quickly in blood and urine (<24 hours; DeGrasse et al., 2014), in fish liver cells with a pH of 7 (over 2 hours; Jackim and Gentile, 1968), and with chlorine treatment (in as little as 15 minutes; Kamp et al., 2016).

2.5. Freshwater Neurotoxin Occurrence

The laboratory studies identifying cyanobacterial species that produce neurotoxins (Tables 2.1 and 2.2) are important to understanding how those neurotoxins are produced and released in the natural environment. However, with all the complexities of a natural system, an examination of anatoxin-a and saxitoxin occurrence in freshwater environments was warranted (Tables 2.3 and 2.4). Only studies that included field collections and toxin analysis are included in Tables 2.3 and 2.4. Studies were excluded if samples of cyanobacteria were collected from a waterbody and exposed to various controlled conditions to induce toxin production in a laboratory. The studies listed are not intended to be exhaustive, but rather were selected because they addressed anatoxin-a, saxitoxin, and related toxin occurrence in different types of freshwater systems throughout the world.

Table 2.3. Examples of literature on anatoxin-a occurrence in freshwater systems based on field collection.

Authors (date)	Study Area	Dates of Collection	Concentration Range[1]
Park et al. (1993)	4 lakes (Japan)	1988-1992	<MDL - 16.3 μg/L
Ballot et al. (2004)	3 lakes (Kenya)	2001-2002	9 - 223 mg/g
Boyer (2008)	140 New York state lakes (USA)	2000-2004	<MDL - 1.0 μg/L
Graham et al. (2010)	23 midwestern lakes (USA)	2006	<MDL - 9.5 μg/L
Al-Sammak et al. (2014)	34 Nebraska reservoirs (USA)	2004-2010	<MDL - 35.7 μg/L
Hilborn et al. (2014)	Lakes in Ohio (USA)	2009-2010	<MDL - 15.0 μg/L
Perri et al. (2015)	Great Lakes (USA)	2011-2012	<MDL - 3.1 μg/L
Shams et al. (2015)	Lake Garda (Italy)	2014	<MDL - 11.3 μg/L
Srivastava et al. (2015)	4 rivers, 10 reservoirs (S. Korea)	1992 - 2004	0.08 μg/L
Vehovszky et al. (2015)	Fancsika pond (Hungary)	2012	NA
McAllister et al. (2018)	8 rivers (New Zealand)	2014-2015	0.008 - 662.0 mg/kg
Salmaso et al. (2016)	4 lakes in southern Alps (Italy)	2014	1.42 - 154 μg/L
Chernova et al. (2017)	freshwaters (Russia)	2013-2015	<MDL - 35 μg/g
Aguilera et al. (2018)	63 waterbodies (Argentina)	1944-2014	up to 6.6 ng/L
Fastner et al. (2018)	Lake Tegel, Berlin (Germany)	2017	943 – 1,870 μg/L

[1]Several studies reported non-detectable (ND) levels of the toxin. However, some of these studies used different minimum detection levels (MDLs). For simplicity, all concentrations less than the reporting limit are called MDLs.

Whereas studies of microcystin occurrence are relatively common, studies on neurotoxin occurrence in freshwater environments currently are less abundant. Aguilera et al. (2018) attributed the lack of anatoxin-a and saxitoxin detections in waterbodies in Argentina to the lack of laboratories that analyze toxins other than microcystin. However, from the Argentina study and others (Tables 2.1 and 2.2), it is evident that many waterbodies contain the cyanobacteria species with documented ability to produce anatoxin-a and saxitoxin, but there is a lack of correlation between anatoxin-a or saxitoxin and any one species of cyanobacteria in some studies (Graham et al., 2010; Kaas and Henriksen, 2002).

Table 2.4. Examples of literature on saxitoxin occurrence in freshwater systems based on field collection.

Authors (date)	Study Area	Dates of Collection	Concentration Range[a]
Negri et al. (1997)	Reservoirs, rivers (Australia)	1992 - 1994	50 - 3,400 μg/g
Kaas and Henriksen (2002)	96 ponds and lakes (Denmark)	1994	5.9 - 224.1 μg[b]
Graham et al. (2010)	23 midwestern lakes (USA)	2006	0.02 - 19 μg/L
Belykh et al. (2016)	Lake Baikal (Russia)	2015	0.21 - 293.90 μg/g
Casali et al. (2017)	Itupararanga Reservoir (Brazil)	2011	0.04 - 0.2 μg/L
Trainer and Hardy (2015)	Washington lakes, pond (USA)	Since 2009	0.21 – 193 μg/L
Aguilera et al. (2018)	63 waterbodies (Argentina)	1944-2014	up to 105.33 ng/L
Christensen et al. (2019)	Kabetogama Lake (USA)	2016	<MDL - 0.08 μg/L
Smith et al. (2019)	New York state (USA)	2017	2.58 – 101.25 μg/L[c]

[a]Paralytic shellfish toxins have varying degrees of toxicity; therefore, many studies report concentrations as total saxitoxin equivalents. [b]Reported as saxitoxin equivalents. [c]Reported as total paralytic shellfish toxins.

Although neurotoxins have been detected in natural lakes (Chernova et al., 2017), rivers (Srivastava et al., 2015), reservoirs (Graham et al., 2010), cobbled streams (McAllister et al. 2018), and other freshwater environments, Aguilera et al. (2018) recorded the most frequent toxin occurrence in reservoirs as opposed to other waterbodies. Al-Sammak et al. (2014) appears to support reservoirs as particularly susceptible to high toxin concentrations, summarizing data from reservoirs in Nebraska, USA showing relatively high anatoxin-a concentrations of up to 35.7 micrograms per liter (μg/L), with about 11.9% of samples testing positive. Saxitoxin, as well as anatoxin-a, may be a concern for reservoirs. Negri et al. (1997) detected saxitoxin in 24 of 31 bloom samples throughout Australia. These samples were primarily collected at farm dams and reservoirs. However, the results may be due to the location, landscape, or morphometry of water bodies in this limited number of studies, rather than a function of reservoirs.

Conversely, Graham et al. (2010) studied 23 midwestern USA lakes and reservoirs where the highest anatoxin-a concentration was reported in a natural lake (Clear Lake, Iowa). Anatoxin-a was present in 30% of the waterbodies sampled, including several reservoirs. This high

occurrence rate may indicate that these midwestern USA eutrophic lakes and reservoirs are more susceptible to anatoxin-a, although the sampling for this study, like many cyanobacteria studies, was purposefully biased toward visible accumulations of cyanobacteria.

McAllister et al. (2018) detected anatoxin-a in cobbled river beds in New Zealand. In fact, anatoxin-a was detected at all eight sites sampled (0.008 to 662.5 milligrams per kilogram, [mg/kg] dried weight; Table 2.3). Saxitoxin also has been detected in river beds (Belykh et al., 2016; Smith et al., 2019). The cyanobacterial species in these cases were primarily benthic. Both pelagic and benthic species producing neurotoxins have been reported, with benthic species near shorelines a particular concern when wild and domestic animals are exposed (Aráoz et al., 2010; Belykh et al., 2016).

Belykh et al. (2016) collected benthic samples in addition to samples from the surface of diseased sponges, finding the presence of the gene capable of producing saxitoxin. Benthic samples were the primary focus of a study on Butterfield Lake (New York, USA; Smith et al., 2019). Two sites, a dock site and a channel site, were identified with *Microseira wollei* (formerly called *Lyngbya wollei*, and identified as a saxitoxin producer, Table 2.2). *Microseira wollei* mats were found on rocks, substrate, aquatic vegetation, and in detached, floating clumps at the dock site, compared with only substrate at the channel site. Interestingly, the saxitoxin concentrations also were substantially higher at the dock site. Yet at both sites, the highest concentrations occurred later in the season when the water was colder, unlike many pelagic cyanobacterial bloom events. Whereas microcystin concentrations were closely related to nitrogen concentrations, paralytic shellfish poisons (e.g., saxitoxin) were more closely related to phosphorus.

Lake morphometry also may be a factor in neurotoxin occurrence. Chernova et al. (2017) examined toxin concentrations in lakes containing toxin-producing cyanobacteria throughout Russia and only found anatoxin-a in lakes in northwestern Russia, notably two shallow lakes; the shallowness may be an important factor as to which toxins are produced. Shallow, polymictic, and polytrophic lakes have been reported as particularly susceptible to cyanobacteria production (Brasil et al., 2016; Christensen and Maki, 2015; McCarthy et al., 2009; Mischke, 2003; Paillisson and Marion, 2011), and an increase in cyanobacteria occurrence can ultimately lead to higher toxin levels, although little research has been done on the specific mechanisms that would cause toxin production or release in shallow water bodies or that would indicate that a shallow lake is susceptible to one particular toxin over another.

The studies reviewed (Tables 2.3 and 2.4) indicate there may be many other factors (altitude, latitude, sample depth, seasonality, and trophic state) that may affect the occurrence and detection of neurotoxins and the cyanobacteria that produce them, but these factors may not consistently predict where neurotoxins occur. For example, *Tychonema bourrellyi*, an anatoxin-a producer, has been traditionally recorded in more northern climates (Butterwick et al., 2005; de los Rios et al., 2004; Watson and Kling, 2017). However, Salmaso et al. (2016) documented *Tychonema bourrellyi*, anatoxin-a, and homoanatoxin-a in Lake Garda in the southern Alps where its presence was not anticipated. The authors correlated depth of distribution of *Tychonema* and found a distinct localized distribution between 10 and 30 m (the euphotic depth) for *Tychonema*, as well as for anatoxin-a and homoanatoxin-a. After examining 36 strains of *Tychonema*, the authors noted that certain strains are restricted to certain habitat types, and that cyanobacteria strains and the neurotoxins they produce can spread to places they have not existed previously as conditions change.

Temporal changes in the composition of cyanobacteria are expected (Becker et al., 2010; Christensen et al., 2019; Watson and Kling, 2017), and thus neurotoxin production is expected to vary temporally. For example, *Tychonema* bloomed primarily in the spring and early summer in the southern Alps (Salmaso et al., 2016; Shams et al., 2015), whereas lakes at higher latitudes may have different seasonal patterns (e.g. Graham et al., 2010), with toxins typically reported in late summer.

Few studies measured more than one or two toxins (Table 2.3 and 2.4), but Park et al. (1993) noted simultaneous detection of microcystin and anatoxin-a. Moreover, two studies noted that peak neurotoxins concentrations (anatoxin-a or saxitoxin) did not coincide with peak microcystin concentrations (Boyer, 2008; Christensen et al., 2019), which has important implications for the many studies that only test for microcystins. Consider samples collected from lakes that provide drinking water to over 22 million people in New York, USA (Boyer, 2008). Although anatoxin-a was identified in 4% of samples as opposed to 50% for microcystin, given the higher toxicity of anatoxin-a and the sheer number of people that could potentially be affected, it would be imprudent to leave out neurotoxin analyses. Hilborn et al. (2014), reporting on disease outbreaks in the USA, noted that whereas most occurrences were due to microcystin, both anatoxin-a and saxitoxin were reported at concentrations of up to 0.09 μg/L for saxitoxin and 15.0 μg/L for anatoxin-a.

In addition to the concern with neurotoxins in drinking water, recreation may be a particular concern when peak toxin concentrations coincide with peak recreational use. For example, Perri et al. (2015) observed anatoxin-a primarily in the late summer in Lake Ontario. Another concern for recreational use is the visibility of blooms. While investigating several dog deaths, Fastner et al. (2018) determined that anatoxin-a was produced when no cyanobacteria

were visible at the surface or in blooms. Studies like these can have important implications for resource management, especially if the season of detection coincides with the season of highest use.

The studies reviewed indicate that anatoxin-a and saxitoxin have widespread distribution, occurring on every continent except Antarctica, but the limited number of studies (in comparison to studies on microcystin) and difference in global distribution may be due to the lack of laboratories performing the analyses for anatoxin-a and saxitoxin and the prohibitive cost of analysis. Distribution differences (e.g., natural lakes versus reservoirs) may be due to the morphometry of the water bodies, or location and landscape rather than a function the water body. Regulatory testing for microcystin without testing for neurotoxins can be a concern for human health, especially in areas where water is used for drinking. Neurotoxin occurrence in recreational waters also is a concern, particularly when peak toxin concentrations coincide with peak recreational use and benthic neurotoxin producing species near shorelines may be a concern when wild and domestic animals are exposed.

2.6. Health Effects in Humans and Other Animals

In humans, poisonings attributed to cyanotoxins have been recorded as far back as the Han dynasty (206 BCE–220 AD) in China (Chorus and Bartram, 1999). Acute and chronic health effects of cyanotoxins have included visual disturbances, vomiting, and acute liver failure (Carmichael et al., 2001), as well as dermatologic, gastrointestinal (Chorus and Bartram, 1999), respiratory (Falconer, 1996), and neurologic symptoms (Al-Sammak et al., 2014; Aráoz et al., 2010; Carmichael and Boyer, 2016). Children are particularly at risk because of their less predictable behavior (playing and splashing in greenish water, for example) and lower body

weight, in addition to the concerns that the toxins could affect growth and development (Weirich and Miller, 2014).

2.6.1. Acute Effects of Freshwater Neurotoxins

More recently, poisonings attributed specifically to neurotoxins have been documented (Wood, 2016). For anatoxin-a, acute health effects of poisoning include convulsions, muscular twitching, imbalance, paralysis, and respiratory failure (Al-Sammak et al., 2014; Rutkowska et al., 2019). Only one coroner-confirmed human death from anatoxin-a exposure (Behm, 2003; Weirich and Miller, 2014) was found in the literature, but that death came 48 hours after the initial exposure, whereas animal deaths have been reported within minutes or hours of exposure (Carmichael and Boyer, 2016), indicating that either the death had a different cause or that there are still questions concerning acute toxicity in humans. Acute health effects from saxitoxin exposure have been primarily documented in marine environments from eating contaminated seafood and include burning, numbness, vomiting, diarrhea, excessive perspiration, salivation, and headache (O'Neill et al., 2016; Rutkowska et al., 2019). One might expect similar responses to saxitoxin in freshwater environments. Although deaths have been reported from exposure to saxitoxin in marine environments, no fatalities have been reported from freshwater saxitoxin exposure (O'Neill et al., 2016).

The dose of the neurotoxin and an individual organism's physiology and behaviors may affect the health impact of exposure. The lethal dose (LD50) of anatoxin-a from mouse toxicology studies is 200–250 μg/kg body weight via intraperitoneal (i.p.) injection (as reported by Carmichael et al., 1990). The saxitoxin LD50 via i.p. injection is 5.5–10 μg/kg body weight (as a rat bioassay as summarized by Chorus and Bartram, 1999). However, the lethal oral dose for both toxins is greater than the i.p. dose (Chorus and Bartram, 1999), and the oral route is the

likely exposure route for humans from drinking water or recreation. In mice, an oral LD50 >5,000 μg/kg body weight for anatoxin-a has been reported (Chorus and Bartram, 1999; Funari and Testai, 2008; Stevens and Krieger, 1991b). Oral dose for saxitoxin in humans ranges from 7–15 μg/kg (as reported by Geraci et al., 1989). Most toxicity studies are on mice or rats, and information on larger mammals and translation of that data to human effects is limited.

Backer et al. (2015) reported on data collected from 2007 through 2011 as part of the Harmful Algal Bloom-related Illness Surveillance System. Fifteen USA states contributed cyanotoxin and human and animal illness data, primarily from freshwater bodies (77%), revealing 3,194 illness events occurred when cyanotoxins were present. Anatoxin-a was detected for 234 (8%) freshwater events, whereas saxitoxin detected for 296 (9%) freshwater events. Although most human exposures were related to consumption of contaminated seafood, 176 exposures that resulted in illness were related to freshwater exposures to cyanobacteria, primarily through recreational activities. Twenty-seven of these cases had associated cyanotoxin data and of these 27, anatoxin-a was detected in 22 cases (81%). Notably, only 15% of the 176 freshwater exposures had cyanotoxin data, but the toxin with highest rate of detection (anatoxin, 81%) is rarely monitored.

Neurotoxins attract attention due to incidents of pet poisoning. Boyer (2008) studied several recreational lakes and reported several dog deaths due to anatoxin-a poisoning in 1998–1999. Dogs are particularly susceptible to ingesting cyanobacteria when they lick their fur to clean themselves after exiting an affected water body (D'Anglada et al., 2015). As such, cyanobacteria have been blamed for dog deaths in the USA (Heiskary et al., 2014), Scotland (Edwards et al., 1992), New Zealand (Wood et al., 2007), and France (Cadel-Six et al., 2007; Gugger et al., 2005a). Backer et al. (2015) noted that 57% of dog poisonings in their review

study were fatal, with anatoxin-a identified as the cause in 18% of cases. Unlike human poisonings, dogs poisoned by anatoxin-a have been confirmed by showing the toxin in stomach contents (Edwards et al., 1992). Many dogs died after eating decaying cyanobacteria on a lakeside where anatoxin degradation products were identified (Hamill, 2001), indicating that presumably anatoxin-a was the toxin responsible for the deaths. Poisonings of cats (Vehovszky et al., 2015), livestock (Al-Sammak et al., 2014; Carmichael and Gorham, 1978), and wildlife (Rose, 1953) have been attributed to toxic cyanobacterial blooms as well.

Wildlife deaths are difficult to study, particularly single animal incidents, due to a variety of reasons including remoteness and predation. Additionally, many incidents are never attributed to cyanotoxins, much less a specific toxin, such as anatoxin-a or saxitoxin. However, numerous animals have been affected by mass mortality incidents linked to blooms, some specifically to neurotoxin exposure. Early reports of dead and dying fish, birds, squirrels, muskrats, skunk, and mink near cyanobacterial blooms in Iowa (USA) lakes were followed by administration of cyanobacteria to laboratory animals that resulted in paralysis and loss of motor function (Rose, 1953), similar to that described for anatoxin-a (Bruno et al., 1994). Anatoxin-a and other cyanotoxins have been suggested in mass lesser flamingo (*Phoeniconaias minor* Geoffroy) deaths in Kenya (Ballot et al., 2004), fish and waterfowl deaths in Russia (Stepanova et al., 2018), and fish on the shore of a lake in Hungary with an anatoxin-a like substance isolated from a nearby bloom (Vehovszky et al., 2015). Saxitoxin mass mortality events also have been reported, including the mass mortality of endemic sponges in Lake Baikal (Belykh et al., 2016) and of fish in Greece (Moustaka-Gouni et al., 2009).

2.6.2. Chronic and Sublethal Effects of Freshwater Neurotoxins

Sublethal effects are those that have any effect other than lethal; these may be either acute (severe and immediate) or chronic (persistent) and are hard to attribute to any one cause. Some acute sublethal symptoms, such as vomiting and diarrhea, have already been covered; other sublethal cyanotoxin effects in humans and domestic animals are difficult to assess. Health problems can have symptoms similar to flu (Carmichael and Boyer, 2016), such as fever, headache, and gastrointestinal distress (Hilborn et al., 2014). In one domestic animal poisoning case, salivation, labored breathing, loss of bodily function, and recumbency were observed in sows and pigs; subsequent tests showed these animals had been exposed to anatoxin-a (Cook et al., 1989).

Sublethal and chronic effects on wildlife and aquatic organisms are even more difficult to study than those in humans and domestic animals, although laboratory studies on steer, rats, mice, birds, and fish (Bruno et al., 1994; Cook et al., 1989; Lopes et al., 2017) have given some indication of what the exposure effects might be in other animals. For example, Bruno et al. (1994) reported the effects of anatoxin-a in mice, including numbness, relaxation of motor function, drowsiness, and respiratory difficulties. Ducks dosed with anatoxin-a experienced diarrhea, tremors, loss of body control, labored breathing, and wing and leg paralysis (Cook et al., 1989). The zooplankton *Daphnia magna* experienced behavioral disturbances (e.g. abnormal circular swimming) and physiological disturbances (changes in heart rate and oxygen consumption) from a range of anatoxin-a doses (Bownik and Pawlik-Skowrońska, 2019), whereas *Daphnia similis* exhibited similar disturbances after exposure to a saxitoxin-producing strain of cyanobacteria (Ferrão-Filho and da Silva, 2020). Saxitoxin exposure studies summarized by O'Neill et al. (2016) showed effects ranging from altered hatching time in fish to

DNA damage. In another laboratory study, swimming behavior of fish changed after a sublethal dose of saxitoxin (Lopes et al., 2017).

Acute toxicity effects of the neurotoxins have been documented as well as more obvious sublethal effects. However, the neurotoxins could have unknown sublethal effects. Whereas a few researchers have studied obvious sublethal effects (e.g., paralysis, loss of motor function), more subtle sublethal effects of the neurotoxins, such as growth, reproductive success, and behavior, are understudied in freshwater systems, and carcinogenic effects of long-term chronic exposure have received little attention.

2.7. Ecosystem Effects of Cyanobacteria and Neurotoxins

Although sublethal effects due to neurotoxin exposure have received less attention than large mortality events, they may be important indicators of ecosystem health. Cyanobacteria are important components of freshwater ecosystems, not only in producing oxygen through photosynthesis (Hudnell, 2008), but also serving as food for planktivores (Paerl and Paul, 2012) and water birds, and forming symbiotic relationships with animals and plants (D'Anglada et al., 2015). Cyanotoxins can affect trophic levels from bacteria to fish, with potential to move up the food chain (Christoffersen, 1996). However, increases in cyanobacteria biomass and their toxins threaten the sustainability of freshwater ecosystems (Hudnell, 2010), and there is evidence that freshwater cyanobacteria and toxins can affect marine ecosystems through downstream transport (Preece et al., 2017).

The ecosystem effects of cyanobacteria on water bodies include effects on nutrient cycling, resilience, and regime shifts in lakes (Cottingham et al., 2015). Cyanobacteria that can fix nitrogen gas are a potential source of additional nitrogen to a lake, allowing cyanobacteria to proliferate even when nutrients are scarce. Some cyanobacteria can access phosphorus in bottom

sediments and both nitrogen and phosphorus can then be released to the water column through phytoplankton mortality, increasing nutrient availability for other phytoplankton (Cottingham et al., 2015).

In terms of the effects of neurotoxins, a few researchers have considered their potential to affect ecosystems (Belykh et al., 2016; Casali et al., 2017; Kaas and Henriksen, 2002). However, the ecological function of these neurotoxins is unknown (Casali et al., 2017) and specific research into sublethal ecosystem effects of neurotoxins is sparse, leaving a broad field for potential research.

2.7.1. Neurotoxins in Substrates and Soils

Potential for cyanotoxins to accumulate in bottom sediment and subsequent absorption onto sediments has been suggested as an important pathway for anatoxin-a elimination from a water body (Klitzke et al., 2011; Rapala et al., 1994). The research specific to neurotoxins in soil is scarce. However, research on microcystins suggests that bottom sediment may act as a reservoir for toxins that later diffuse into the water column (Zastepa et al., 2015), and an increase in microcystin concentrations during and immediately after internal phosphorus loading from sediments demonstrates this possibility (Miller et al., 2017; Orihel et al., 2015). Resuspension of sediments by fish may also re-introduce toxins into the water column (Huisman et al., 2018). In an anatoxin-a study, adsorption depended primarily on the texture of the sediment, bonding weakly to sandy sediments and more strongly to clay sediments (Klitzke et al., 2011). Because the mechanism of anatoxin-a sorption is primarily by cation exchange, desorption of anatoxin-a is enhanced by conditions such as increasing pH (Klitzke et al., 2011). The authors reported that anatoxin-a sorption exceeds that of other cyanotoxins (cylindrospermopsin, microcystin, and

nodularins)—the ecological significance being that it may decrease the availability of anatoxin-a to microbes and thus lead to longer degradation times in sediment (Klitzke et al., 2011).

Burns et al. (2009) reported that saxitoxin-producing cyanobacteria can settle into sediments and remain viable for years, and in laboratory experiments, saxitoxin removal from the water column to sediments was rapid and significant (>50%), adsorbing to clays and sediment more strongly in freshwater environments than in seawater (9:1). The authors reported that saxitoxin release from sediments was greater in freshwater environments than in seawater. Another possible concern is the transformation to more toxic variants. Kayal et al. (2008) showed that microbial treatment of saxitoxin resulted in a decrease in less toxic variants (C-toxins) and an increase in more toxic variants (gonyautoxins). Therefore, in addition to the sediment being a sink, sediment could be a source, particularly when freshwater sediments are disturbed after strong winds or storms, bioturbation from fish, or dredging. Belykh et al. (2016) suggested that saxitoxin exposure likely affects other substrates, including sponges, and considered the alarming sudden and mass mortality of sponges co-occurring with filamentous cyanobacteria in Lake Baikal an "ecological crisis."

The potential exists for cyanotoxins to be transported and accumulate in terrestrial soils as well, affecting microbial processes, and in time could be transported back to water bodies (Bouaïcha and Corbel, 2016). Cyanotoxins are transported to terrestrial soils through agricultural application of fertilizers or sewage sludge (Ai et al., 2020) that contain cyanobacteria, through cyanotoxin-contaminated water used for irrigation (Bouaïcha and Corbel, 2016), and possibly through aerosolized particles transported to terrestrial environments by the wind (Grattan et al., 2016). The effects of cyanotoxins in sediment are poorly understood, partially due to limited research, particularly for neurotoxins, and limited analytical methods for toxins in sediment.

2.7.2. Effects on Aquatic and Terrestrial Plants

Plants are important to aquatic systems, filtering out cyanotoxins and other contaminants through biological transformation (Nimptsch et al., 2008); however, cyanotoxins can affect the growth of macrophytes (Christoffersen, 1996). Mitrovic et al. (2004) reported that exposure of aquatic plants (macrophytes and macroalgae) to anatoxin-a at concentrations between 5 and 25 μg/mL (5,000 and 25,000 μg/L) reduced photosynthetic oxygen production. However, these concentrations are greater than the highest concentration reported per volume found for this literature review (1,870 μg/L; Fastner et al., 2018; Table 2.3), which may indicate that the concentrations needed to cause negative effects are much greater for plants than for animals, but another study showed environmentally relevant concentrations of anatoxin-a (15 μg/L) produced phytotoxic effects in submerged vegetation (*Ceratophyllum demersum*) over the course of 2 weeks, and various morphogenic effects and a decline in total biomass was observed during longer periods up to 8 weeks (Ha and Pflugmacher, 2013).

There is potential for cyanotoxins to be transported and accumulate in terrestrial soils, leading to absorption by terrestrial vegetation (Bouaïcha and Corbel, 2016). Mitrovic et al. (2004) reported on recent studies showing that microcystins inhibited growth of seedlings in terrestrial crops, and similar results might be expected for the neurotoxins, although they have different modes of action than microcystin. Corbel et al. (2014) reviewed cyanotoxins introduced to the terrestrial soil ecosystem after irrigating crops and suggested that the introduction of neurotoxins could modify ion transport in plant cells and may lead to bioaccumulation of toxins that would result in a potential adverse health effect for the humans and animals who consume these plants.

2.7.3. Effects on Lower Trophic Levels

The effects of cyanotoxins on fish, birds, and mammals have been noted, but they also can affect lower trophic levels, which include bacteria, protozoans, and zooplankton (Christoffersen, 1996; Holland and Kinnear, 2013). Li et al. (2015) reported that primary producers, such as algae and cyanobacteria, differ in nutritional content. When cyanobacteria or other lower food-quality algae dominate, it can have adverse effects throughout the food web. Toxin production may be a mechanism for cyanobacteria to reduce competing grazer communities, which in turn can help sustain the cyanobacteria population. Although the authors did not address neurotoxins specifically, they showed that the dominance of toxin-producing cyanobacteria leads to the failure of normal predator-prey relationships, increasing the transfer of nutrients to sustain the toxin-producing cyanobacteria over freshwater algae. A study on microcystin (Vanderploeg et al., 2001) showed that zebra mussels (*Dreissena polymorpha*) preferentially expel *Microcystis aeruginosa* while ingesting other algae, thereby increasing biomass of *Microcystis aeruginosa* in the water column. Similar studies might help us understand whether this preferential treatment is true of neurotoxins as well.

Christoffersen (1996) reported that some protozoa are able to digest *Microcystis aeruginosa* (an anatoxin-a producer), although most mesozooplankton avoid ingestion of toxic species. The mesozooplankton *Daphnia* coexists during toxic blooms, possibly having a resistance to toxins or switching to a non-toxic food source when necessary. Microzooplankton (such as rotifers) can survive off toxic strains of *Microcystis aeruginosa*, although anatoxin-a (0.2–5 μg/L) affected reproduction in several microzooplankton species. Christoffersen (1996) concludes that some phytoplankton and protozoans show effects (such as inhibited growth, reduced grazing, failure to thrive, or bioaccumulation) at much lower cyanotoxin concentrations

than fish, birds, or mammals. Despite these exposures and effects, very little research exists on the effects of neurotoxins on lower trophic levels.

2.7.4. Bioaccumulation

Bioaccumulation of neurotoxins may be an important route of exposure to larger organisms, including humans. Al-Sammak et al. (2014) examined the potential for anatoxin-a bioaccumulation in aquatic plants and animals, and whereas anatoxin-a was not detected in any fish sampled in that study, biomagnification of some toxins may be an important consideration for human exposure. In another study of European lakes (Pawlik-Skowrońska et al., 2012), anatoxin-a was determined to bioaccumulate in fish, although concentrations of anatoxin-a in the fish livers were higher than in fish tissue, which may be an indication that anatoxin-a bioaccumulation may be more of a concern for wildlife that eat whole fish than in humans.

In the marine environment, saxitoxins are well-known to be ingested and concentrated by fish and shellfish (Carmichael et al., 1985; Cusick and Sayler, 2013), but also have been shown to accumulate in freshwater fish and shellfish (Calado et al., 2019; Giovannardi et al., 1999). Conversely, another study showed a bioaccumulation of microcystin in fish tissue, but no neurotoxins were detected (Hardy et al., 2015), indicating that bioaccumulation may be dependent on fish species. Negri and Jones (1995) suggested that because saxitoxin-producing cyanobacteria are present in many freshwater environments, there may be ecological effects throughout the food web, including effects at higher trophic levels.

The results of the few studies on bioaccumulation indicate that further studies are necessary to add to our basic knowledge on the ecological impact of anatoxins and saxitoxins and their fate in the aquatic environment. Furthermore, additional studies at lower trophic levels and symbiotic relationships of the cyanobacteria and their toxins with plants and animals would

help in the understanding of the complex pathways a toxin can move through the environment. The sudden and mass mortality events described as a result of cyanotoxin exposure, may have overshadowed less newsworthy non-lethal effects of these powerful freshwater neurotoxins.

2.8. Summary

The aim of this review was to synthesize the findings of individual studies that examined anatoxin-a and saxitoxin, in order to report on the effects of these neurotoxins to humans, animals, and freshwater ecosystems. Toxic cyanobacteria are a growing concern worldwide because of their negative effects. However, research on the occurrence of the neurotoxins anatoxin-a and saxitoxin in freshwater systems is relatively scarce when compared to research on other cyanotoxins, especially microcystin.

Cyanotoxin research is a growing field and the tables of literature summarized allow for an examination of freshwater settings where anatoxin-a and saxitoxin have been reported. Examples of human and animal health concerns can range from acute to chronic. However, few researchers studied chronic or sublethal effects of the neurotoxins. Ecosystem health also is a concern, as the effects of toxicity may be far reaching and include consequences throughout the food web.

The neurotoxins anatoxin-a and saxitoxin are produced globally by many freshwater cyanobacteria. Neurotoxins produced by cyanobacteria could be an important part of freshwater ecosystem function. However, the current research has not determined why these neurotoxins are produced, and our understanding is limited in terms of what triggers their production and release and what effects, particularly sublethal effects, they have on freshwater ecosystems. For resource managers, implications include consequences of issuing advisories based on the presence of cyanobacteria, without confirmation of toxins. However, given the acute animal poisonings that

have been linked to these toxins, the limited knowledge of their function and effects could have serious and lasting implications for individual organisms and entire ecosystems. The possibility of fatal poisonings in freshwater environments amplifies the need for additional research on anatoxin-a and saxitoxin. The lack of particular freshwater systems in the literature, in combination with known toxic events, may be an indication of where additional research on anatoxin-a or saxitoxin may be appropriate. The growing concern over cyanotoxins will require further study of neurotoxins such as anatoxin-a and saxitoxin, their occurrence and biogeography, triggers of production and release, environmental fate and degradation, primary and secondary exposure routes, diurnal variation, food web effects, the effects of cyanotoxin mixtures, and sublethal health effects on individual organisms and populations.

CHAPTER 3: PHYTOPLANKTON COMMUNITY AND ALGAL TOXICITY AT A RECURRING BLOOM IN SULLIVAN BAY, KABETOGAMA LAKE, MINNESOTA, USA[2]

3.1. Introduction

Cyanobacteria, often referred to as blue-green algae, are the oldest oxygen-producing organisms (Schopf, 2002) and have had major effects on the Earth (Paerl and Paul, 2012). They provide food for fish and are important for multiple trophic levels but can be harmful to ecosystems by contributing to hypoxia, disrupting the food web, and releasing toxins.

Specific cyanobacteria have traits that allow them an advantage over other prokaryotic cyanobacteria and eukaryotic algae, and responses to environmental conditions can be taxon-specific (Beaver et al., 2018). For example, *Anabaena* can fix nitrogen from the atmosphere (Stewart, 1973) and are adapted to relatively high light environments and conditions. Conversely, *Aphanizomenon* are adapted to relatively low light conditions (De Nobel et al., 1998), and *Planktothrix* can withstand continuous mixing in shallow lakes (Mischke, 2003). Additionally, cyanobacteria size and density can affect buoyancy (Reynolds et al., 1987). *Microcystis*, for example, accumulates carbohydrates to regulate density as a type of "ballast" mechanism (Reynolds et al., 1987). These competitive advantages are a key to understanding dominant forms of phytoplankton during the formation of blooms—the visible and rapid growth of cyanobacteria that typically occurs at the water surface.

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[2] The material in this chapter was co-authored by Victoria Christensen, Ryan Maki, Erin Stelzer, Dr. Jack Norland, and Dr. Eakalak Khan. Victoria Christensen had primary responsibility for the literature research, data analysis, statistics, writing the draft, and all revisions of this chapter. Ryan Maki provided field logistics and proofreading. Erin Stelzer performed the laboratory analysis and provided proofreading. Victoria Christensen is the primary developer of the conclusions and research gaps that are advanced here, and Dr. Jack Norland and Dr. Eakalak Khan proofread and served as advisors.

The toxins in these cyanobacterial blooms (cyanotoxins) can adversely affect humans, animals, and ecosystems. Whereas short-term human health responses to cyanobacterial toxin exposure are known (Dow and Swoboda, 2000; Hilborn et al., 2014), long-term effects of human or animal exposure, as well as ecosystem effects are largely unknown. This gap in our scientific knowledge is a concern in areas with recurring blooms. Moreover, geographical distribution of cyanotoxin occurrence from existing literature indicates a substantial data gap in a large expanse of the north central USA (Svirčev et al., 2019). Therefore, this part of the dissertation research examined the temporal variability of the phytoplankton community and the relation to cyanotoxins at a remote recurring bloom site in Sullivan Bay, Kabetogama Lake, Voyageurs National Park.

Many conditions in combination, such as nutrients from human activities (Paerl and Paul, 2012), agricultural practices (Paerl, 1988), or lower salinity levels (Li et al., 2015), can set up a water body to be susceptible to bloom formation. However, with the exception of internal loading at various locations in Kabetogama Lake (Christensen et al., 2013) and the extensive clay soil deposits on the lake's south side (Kallemeyn et al., 2003) that could account for high concentrations of nutrients in the lake, many of the nutrient sources common to lakes with frequent algal blooms are absent from Voyageurs National Park due to its remote location. Therefore, there is a unique opportunity to evaluate seasonal changes in phytoplankton composition as it relates to toxin production in an area without the substantial urban and agricultural nutrient sources. Moreover, Voyageurs National Park is in an area without significant cyanotoxin peer-reviewed literature (Svirčev et al., 2019). The results may be useful to similar remote or northern temperate lakes experiencing annual algal blooms.

3.1.1. Background

Cyanobacterial algal blooms in Kabetogama Lake, one of more than 30 lakes in Voyageurs National Park, have frequently produced the toxin microcystin (Christensen et al., 2011). During a 2008-09 study at Voyageurs National Park, microcystin (total microcystin and nodularin expressed as relative amount of microcystin-LR) was reported in 11 of 14 bloom samples (78%; Christensen et al., 2013), and 7 of 14 bloom samples (50%) exceeded the 1-μg/L guideline for drinking water established by the World Health Organization (WHO; World Health Organization, 2003). Two samples exceeded 20 μg/L, putting them into the WHO high-risk category for recreational exposure (Chorus and Bartram, 1999). Moreover, Voyageurs National Park lacked data for other algal toxins, such as anatoxin-a and saxitoxin. The WHO recommends changes in monitoring for cyanobacterial blooms with microcystin-LR concentrations that exceed 10 μg/L. The WHO also recommends a moderate health alert when concentrations exceed 20 μg/L in recreational water (Chorus and Bartram, 1999).

Substantial biovolumes of two cyanobacteria known to produce toxins, *Dolichospermum* and *Microcystis*, have composed much of the phytoplankton community in Kabetogama Lake blooms (Christensen et al., 2011). *Dolichospermum*, *Microcystis*, and *Planktothrix* are all commonly known to produce microcystins (Rantala et al., 2006), whereas anatoxin-a and saxitoxin are known to be produced by additional species, such as *Aphanizomenon*, *Lyngbya*, or *Cylindrospermopsis* (Chorus and Bartram, 1999; Graham et al., 2010).

Production of microcystin can only occur if the microcystin synthetase (*mcy*) gene is present in the genome (Sipari et al., 2010). Individual strains within a microcystin-producing genus may include both the toxic strains and nontoxic strains, in other words with or without the *mcy* genes, respectively (Rantala et al., 2006). These toxic and nontoxic strains can be

differentiated in about 3 hours by quantitative polymerase chain reaction (qPCR), a molecular detection method (Francy et al., 2015; Sabart et al., 2015).

The purpose of this portion of the dissertation research was to better understand changes throughout the open water season in toxin concentrations and cyanobacterial community structure (in terms of abundance, species composition, and toxin production capability). Specific objectives are to (1) identify and quantify toxin producing cyanobacteria with molecular tools (qPCR); and (2) determine the seasonal changes in toxin production and changes in phytoplankton community structure.

The novelty of this study is that it included the collection of samples after ice out occurred and before any algal blooms were visible, whereas most studies only collect these types of samples when algal blooms are visible. Additionally, anatoxin-a and saxitoxin, two toxins that understudied in freshwater, are covered by only 9% and 27% of published cyanotoxin papers, respectively (Svirčev et al., 2019), and this research at Voyageurs National Park will provide more understanding on these toxins. The knowledge gained from this study may be valuable to other researchers and resource managers, who want to better predict, manage, and mitigate the occurrence of toxic cyanobacterial blooms.

3.1.2. Site Description

Kabetogama Lake lies within Voyageurs National Park, but about 20 km of private shoreline surrounds the south end of the lake (Kallemeyn et al., 2003). Kabetogama Lake is 10,425 ha, with a watershed to lake ratio of 196.7, a maximum depth of 24.3 m, and a mean depth of 9.1 m (Kallemeyn et al., 2003). Land use in the Kabetogama Lake watershed is primarily forested (59%), with water features making up another 38%, and grasslands 2% (Kallemeyn et al., 2003). Small pockets of developed areas and agriculture in the form of hay

fields make up less than 1% of the watershed. Kabetogama Lake suffers from annual algal blooms unlike most of the park's lakes, which are primarily oligotrophic to mesotrophic. Moreover, Kabetogama Lake is noticeably different than the other large lakes of the park in that thermal stratification occurs infrequently, whereas thermoclines are typically present throughout the summer in the other large lakes. However, dissolved oxygen concentrations have fallen below accepted standards in Kabetogama Lake when thermoclines developed during hot, still weather (Christensen et al., 2004).

Kabetogama Lake also has different water chemistry than the park's other large lakes (Kallemeyn et al., 2003), and differences in water quality occur between the shoreline, mid-lake, and individual bays (Christensen et al., 2011). Additionally, Kabetogama Lake has higher specific conductance, nutrient, and chlorophyll-*a* concentrations (Kallemeyn et al., 2003) and is shallower than other park lakes, resulting in higher temperatures, which have been connected with algal blooms in other systems (Mantzouki et al., 2018). At least one study points to the importance of local environmental conditions in relation to microcystin production (Singh et al., 2015).

Sullivan Bay within Kabetogama Lake is near the southern border of the park, with depths ranging from 1.8 to 4.9 m (Payne, 1991). Sullivan Bay is characterized by higher dissolved solids and alkalinity than the rest of the lake, reflecting inflow from Ash River (Payne, 1991). A recurring bloom site in Sullivan Bay (USGS site number 482542092493701) was selected for intensive algal bloom sampling and a comparison to phytoplankton species. Sullivan Bay is shallow, polymictic (Kallemeyn et al., 2003; Payne, 1991), and a popular recreational location with cottages and resorts upstream. The degraded water quality in this bay has

historically been attributed to the inflow from Ash River and its rich geological substrates (Kallemeyn et al., 2003), but no recent source allocation has been conducted.

3.2. Methodology

The data presented in this paper were part of a 2-year study (2016-2017); however, this paper covers only 2016 when phytoplankton samples were collected. Twenty-one samples were collected at the Sullivan Bay algal bloom site (482542092493701) between June 12, 2016, and September 13, 2016. All samples were collected between 8 a.m. and 3 p.m. Central Daylight Time. The exact location was sampled, whether or not a bloom was present at the time of sampling, and even when there was an obvious bloom only meters away. In general, samples were collected weekly, until August when samples were collected twice per week on subsequent days. The 21 samples were analyzed for cyanotoxins and cyanobacteria. The weekly samples (12 samples) were analyzed for phytoplankton abundance and community structure. Sampling and field measurements were made according to field methods established during previous studies in Voyageurs National Park (Christensen et al., 2004, 2011) and generally followed standard USGS techniques for lake sampling (U.S. Geological Survey, 2018).

3.2.1. Field Collection and Processing

Phytoplankton, cyanotoxin, and cyanobacterial gene samples were collected from just beneath the water surface with dip sampling procedures (Graham et al., 2008). A 250-mL wide-mouthed sampling bottle was used for the phytoplankton, and a sterile 1-L polyethylene bottle was used for cyanotoxins and cyanobacterial gene samples.

Water samples were processed in a lakeside field laboratory where they were filtered and/or preserved according to USGS methods (Graham et al., 2008; Patton and Kryskalla, 2003). Samples for phytoplankton abundance and community structure were preserved with a 9:1

Lugol's iodine:acetic acid solution and stored in a refrigerator until batch shipment to BSA Environmental Services, Inc. (Beachwood, OH).

An aliquot of water for cyanotoxin (ELISA) analyses was extracted from the 1-L bottle, put into a 125-mL bottle, and stored in a freezer. Water for gene analyses was extracted from the 1-L bottle and filtered onto a 0.4-µm pore-size Nucleopore polycarbonate filter. The volume filtered depended on the clarity of the water, but typically sample volumes ranged from 25-100 mL. Each filter was placed into a 2-mL screw-capped vial with 0.3 grams of acid-washed beads and frozen until batch shipment. Filters were shipped to the USGS Ohio Water Microbiology Laboratory (Columbus, OH) on dry ice in batches for analyses.

3.2.2. Laboratory Analyses

3.2.2.1. Phytoplankton Analyses

Phytoplankton abundance and community structure were analyzed by microscopy for taxonomic identification and enumeration by BSA Environmental Services, Inc. (Beachwood, OH). Phytoplankton were enumerated to the lowest possible taxonomic level using membrane-filtered slides (McNabb, 1960). A minimum of 400 natural units (colonies, filaments, and unicells) was counted from each sample in accordance with Lund et al. (1958). Counting 400 natural units provided accuracy within 90-percent confidence limits.

3.2.2.2. Cyanotoxin Analyses

Cyanotoxins (anatoxin-a, microcystin, and saxitoxin) were analyzed in one batch per toxin at the end of the season at the USGS Ohio Water Microbiology Laboratory. The samples were lysed by three sequential-freeze/thaw cycles followed by syringe filtration with a 0.7-µm glass-fiber syringe filter and analyzed by ELISA kits acquired from Abraxis, LLC (Warminster, PA) for microcystins/nodularins (U.S. Environmental Protection Agency, 2016), anatoxin-a, and

saxitoxins (Graham et al., 2010). The minimum reporting levels (MRL) are 0.3 µg/L as anatoxin-a equivalents, 0.3 µg/L as total microcystin and nodularin expressed as microcystin-LR equivalents (called microcystin throughout Chapters 3 and 4), and 0.02 µg/L as saxitoxin equivalents. Samples exceeding the highest calibration standard were diluted.

3.2.2.3. Cyanobacterial Gene Analyses

Samples were extracted and analyzed for cyanobacterial genes at the USGS Ohio Water Microbiology Laboratory by use of qPCR according to procedures in Stelzer et al. (2013), summarized below. Molecular assays for cyanobacterial genes were analyzed to enumerate the following:

- General cyanobacteria (Rinta-Kanto et al., 2005)
- Genus-specific assays for *Dolichospermum*, *Microcystis*, and *Planktothrix* (Doblin et al., 2007; Ostermaier and Kurmayer, 2009; Rinta-Kanto et al., 2005)
- Genus-specific microcystin (*mcyE*) toxin gene assays for *Dolichospermum*, *Microcystis*, and *Planktothrix* (Rantala et al., 2006; Sipari et al., 2010)
- Saxitoxin (*sxtA*) toxin gene (Al-Tebrineh et al., 2012)
- Anatoxin-a (*anaC*) toxin gene (Sabart et al., 2015)

3.2.2.4. DNA Extraction and qPCR Analyses

Samples were extracted (Stelzer et al., 2013) by use of a DNA-EZ extraction kit (GeneRite, North Brunswick, NJ) according to manufacturer's instructions, except that no prefilter was used and the final elution volume was 150 microliters (µL). An extraction blank was included with each batch of sample extractions.

From each sample extract, 5 µL was analyzed by qPCR in duplicate for each assay described above by using primer and probe sets as well as qPCR performance conditions detailed

in the lysed references for each assay. All assays were performed on either an Applied Biosystems 7500™ or a StepOnePlus™ (Life Technologies, Carlsbad, CA) thermal cycler. TaqMan™ Universal PCR Master Mix (Life Technologies, Carlsbad, CA) was used if the assay included a probe, whereas SYBR™ Green PCR Master Mix (Life Technologies, Carlsbad, CA) was used if the assay did not include a probe. If the sample results were considered inhibited, the extracts were diluted and reanalyzed (Stelzer et al., 2013). Results from diluted reanalyzed samples were used to compute final concentrations.

3.2.2.5. Standard Curves and Quantifying Cyanobacterial Toxin Genes

Standards were included in duplicate with each qPCR run to construct a seven-point standard curve. Plasmid standards for each assay were used to establish standard curves for quantification. Before qPCR analysis, plasmid containing *Escherichia coli* (*E. coli*) was grown overnight and plasmids were extracted and purified from the *E. coli* cells using the QuickLyse Miniprep Kit (Qiagen, Valencia, CA). The copy number of each target was calculated using the DNA concentration measured by the Qubit™ dsDNA High Sensitivity Assay (Life Technologies, Carlsbad, CA) and the molecular weight of the plasmid. Sample results were reported as copies per 100 milliliters (copies/100 mL). Standard curve characteristics for this study are listed in Table 3.1.

Table 3.1. Standard curve characteristics for quantitative polymerase chain reaction (qPCR) analyses performed by the U.S. Geological Survey Ohio Water Microbiology Laboratory during this study of Kabetogama Lake, Sullivan Bay, Northwest, near Ash River, Minnesota (U.S. Geological Survey Site No. 482542092493701), Voyageurs National Park, 2016.

qPCR assay	Dynamic range	Amplification efficiency (percent)	R^2 value	LoD	LoQ
general cyanobacteria	66.0 - 6.60E+07	95 - 97	0.995 - 0.996	26	72
general *Microcystis*	9.44 - 9.44E+06	91 - 96	0.994 - 0.997	27	55
general *Planktothrix*	16.1 - 1.61E+07	99 - 100	0.998 - 0.999	18	34
general *Dolichospermum*	24.6 - 2.46E+07	98 - 100	0.992 - 0.995	230	550
Microcystis microcystin toxin gene (*mcyE*)	11.4 - 1.14E+07	93 - 98	0.997	13	39
Planktothrix microcystin toxin gene (*mcyE*)	28.4 - 2.84E+07	94 - 95	0.993 - 0.998	51	110
Dolichospermum microcystin toxin gene (*mcyE*)	19.2 - 1.92E+07	95 - 96	0.997 - 0.998	46	120
saxitoxin toxin gene (*sxtA*)	30.8 - 3.08E+07	81 - 84	0.995 - 0.998	14	45
anatoxin-a toxin gene (*anaC*)	74.5 - 7.45E+07	83 - 84	0.999	32	60

The limit of detection (LoD) and limit of quantification (LoQ) were calculated for each assay (Francy et al., 2017), in addition to the coefficient of determination (R^2). The LoD and LoQ values were initially cycle threshold values converted to and reported as copies per qPCR reaction by use of an assay-specific standard curve. Sample results lower than the LoQ but above the LoD are reported as estimated values. Because original sample volumes and dilutions to overcome inhibition were often different for each sample or set of samples, the LoD was applied on a sample-by-sample basis to determine sample reporting limits. The sample reporting limits are the "less-than values" for each sample and assay and are also reported as copies/100 mL.

3.2.2.6. Quality Assurance and Quality Control

Quality-control samples, consisting of replicates, blanks, and spikes were analyzed to document the variability associated with sample collection and laboratory procedures. Two field quality-control samples (one replicate and one blank) were collected and analyzed in 2016, which is approximately 16% of the environmental samples. Additional field quality samples

were analyzed at other field sites for the larger study effort, and median relative percent difference for all replicate samples was between 0 and 10 percent (Christensen and Olds, 2019).

Effectiveness of equipment cleaning and sample processing was assessed by laboratory analysis of field blanks. Laboratory blank water was processed in the field with the same collection bottles, filtering devices, and methods as for native water samples. For cyanobacterial gene samples, one additional filter blank using sterile water was filtered each day samples were processed. If detections are found in the blank samples, the limit of blanks is calculated and used in determining the detection limit of an assay. This is defined as the 95th percentile of all cycle threshold values for the blanks for a specific assay.

At the USGS Ohio Water Microbiology Laboratory, laboratory replicates were analyzed with each batch and considered acceptable at a coefficient of variance equivalent to +/- 20% of average or expected value or less. If laboratory replicate samples did not meet this criterion, then the entire plate was reanalyzed. More variation may be expected with field replicates due to difficulty with homogenizing intact cyanobacterial cells when splitting samples; therefore, field replicate samples were not reanalyzed if percent relative standard deviation (RSD) exceeded +/- 20%, but rather accepted as inherent variability due to temporal or spatial variation.

3.3. Results and Discussion

3.3.1. Seasonal Changes in Phytoplankton Community

Twelve samples were collected at the Sullivan Bay algal bloom site (482542092493701) and analyzed for phytoplankton abundance and community structure. Detailed taxonomic identification and enumeration data from these samples were published in the USGS ScienceBase Catalog (Christensen and Olds, 2019).

Total cell abundance and total biovolume (Table 3.2) varied early in the season but had two distinct peaks (August 1, 2016, and August 16, 2016). Cell abundance is the total number of cyanobacterial or algal cells in the sample per unit volume; biovolume indicates algal mass by volume (Francy et al., 2015). In other words, cell abundance and cell biovolume are different because some algal cells are larger than others and, therefore, may have greater biovolume than others. The summer peaks are followed by a rapid decline that continued through Sept. 13, 2016.

In general, the percent cyanobacterial biovolume ranged from 7% in June to nearly 70% in August (Table 3.1). Among the cyanobacteria recorded early in the season are *Dolichospermum*, *Aphanizomenon*, and *Chroococcus* (Fig. 3.1). In late June, *Aphanocapsa*, *Planktolyngbya*, *Planktothrix* (formerly *Oscillatoria*), and *Pseudanabaena* emerge in water samples (Christensen and Olds, 2019). Generally, *Dolichospermum* dominates through mid-July. However, from mid-July to mid-August, samples consist primarily of *Aphanizomenon*, a producer of anatoxin-a and saxitoxin, which peaked on August 16, 2016. In late August, *Planktothrix* and *Dolichospermum* dominated. Finally, in the sample collected in mid-September, *Aphanizomenon* dominated once again.

3.3.2. Seasonal Changes in Cyanobacterial Genes

Both general cyanobacterial counts and genus specific counts generally show a seasonal pattern, with higher counts in late summer (Table 3.3). However, not all genera peaked at the same time. For example, general *Dolichospermum* counts were greatest on August 10. Following a rapid decline in *Dolichospermum*, *Planktothrix* counts were greatest about a week later, on August 16 (Table 3.3). Anatoxin-a toxin genes (*anaC*) and saxitoxin toxin genes (*sxtA*) both peaked on August 16, 2016 (Table 3.4) with a pattern similar to general cyanobacteria and general *Planktothrix* (Table 3.3).

Table 3.2. Summary of phytoplankton abundance and biovolume in water samples from Kabetogama Lake, Sullivan Bay, Northwest, near Ash River, Minnesota (U.S. Geological Survey Site No. 482542092493701), Voyageurs National Park, 2016.

Date	Abundance (cell/L)	Total biovolume (μm^3/L)	Cyanobacteria biovolume percent
6/21/2016	26,156,702	8,087,275,833	7.0
6/27/2016	63,105,589	7,026,359,688	24
7/6/2016	40,101,966	2,554,264,945	30
7/12/2016	85,944,746	8,749,585,308	39
7/19/2016	119,889,980	5,836,783,205	43
7/26/2016	229,360,060	18,597,701,465	53
8/1/2016	286,569,740	19,499,667,005	53
8/10/2016	156,776,705	10,854,356,404	60
8/16/2016	289,829,158	25,872,678,424	67
8/23/2016	282,470,651	11,604,888,019	69
8/30/2016	264,377,500	9,544,215,996	67
9/13/2016	34,668,911	2,050,802,261	12

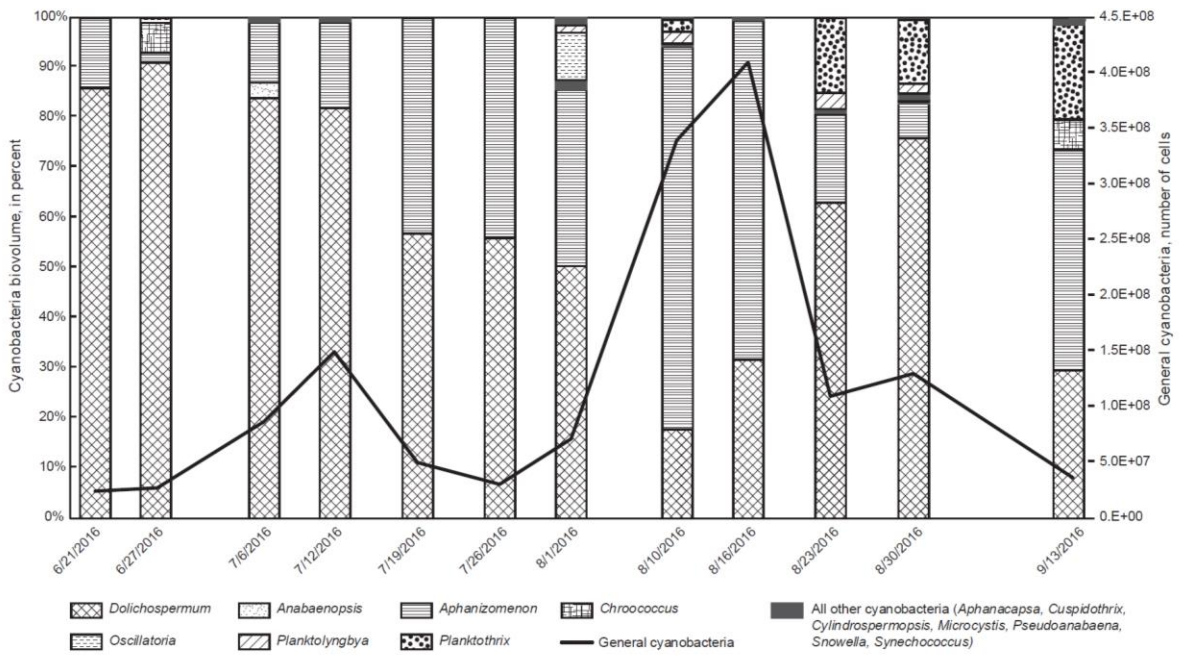

Figure 3.1. Cyanobacteria species as percent total biovolume compared to general cyanobacteria, of samples collected from Kabetogama Lake, Sullivan Bay, Northwest, near Ash River, Minnesota (U.S. Geological Survey site number 482542092493701), Voyageurs National Park, 2016.

Table 3.3. Results of general cyanobacterial gene analysis by quantitative polymerase chain reaction (qPCR) in water samples from Kabetogama Lake, Sullivan Bay, Northwest, near Ash River, Minnesota (U.S. Geological Survey Site No. 482542092493701), Voyageurs National Park, 2016.

Date	General qPCR (gene copies per 100 milliliters)			
	Cyanobacteria	*Microcystis*	*Planktothrix*	*Dolichospermum*
6/21/2016	24,000,000	<810	19,000	500,000,000
6/27/2016	27,000,000	5,000	12,000	1,100,000,000
7/6/2016	87,000,000	2,500	<540	1,400,000,000
7/12/2016	150,000,000	40,000	41,000	20,000,000,000
7/19/2016	50,000,000	38,000	250,000	1,600,000,000
7/26/2016	30,000,000	1,600	160,000	1,500,000,000
8/1/2016	71,000,000	24,000	1,200,000	5,300,000,000
8/10/2016	340,000,000	180,000	34,000,000	120,000,000,000
8/16/2016	410,000,000	210,000	97,000,000	3,500,000,000
8/23/2016	110,000,000	70,000	24,000,000	3,600,000,000
8/30/2016	130,000,000	5,800	41,000,000	2,000,000,000
9/13/2016	33,000,000	E820	11,000,000	55,000,000

Table 3.4. Results of gene specific analysis by quantitative polymerase chain reaction (qPCR) in water samples from Kabetogama Lake Sullivan Bay, Northwest, near Ash River, Minnesota (U.S. Geological Survey Site No. 482542092493701), Voyageurs National Park, 2016.

Date	Genus specific DNA by qPCR (gene copies per 100 milliliters)				
	Microcystis mcyE	*Planktothrix mcyE*	*Dolichospermum mcyE*	Anatoxin-a (*anaC*)	Saxitoxin (*sxtA*)
6/21/2016	<390	E2,800	160,000	<960	E560
6/27/2016	E700	<33,000	410,000	2,100	2,800
7/6/2016	E750	47,000	520,000	3,800	1,600
7/12/2016	10,000	<33,000	740,000	5,200	10,000
7/19/2016	12,000	<33,000	640,000	4,500	41,000
7/26/2016	1,200	<33,000	230,000	4,200	14,000
8/1/2016	13,000	62,000	1,200,000	23,000	160,000
8/10/2016	79,000	1,400,000	1,500,000	43,000	1,100,000
8/16/2016	39,000	37,000,000	420,000	95,000	14,000,000
8/23/2016	13,000	3,200,000	530,000	2,800	440,000
8/30/2016	E640	28,000,000	160,000	1,800	42,000
9/13/2016	E400	3,700,000	<1,400	E1,300	E660

3.3.3. Seasonal Changes in Toxin Production

In addition to examining the potential for toxin production using the qPCR molecular method, samples were analyzed for the cyanotoxins anatoxin-a, microcystin, and saxitoxin by

enzyme-linked immunosorbent assays (ELISA) to obtain concentrations. Toxin analysis by ELISA (Fig. 3.2), resulted in lower microcystin-LR concentrations than previous sampling at Kabetogama Lake (Christensen et al., 2013); however, previous samples were generally collected during active blooms. Microcystin-LR was detected in about 58% of samples (7 of 12). Anatoxin-a and saxitoxin detections were infrequent, with anatoxin present in 1 of 11 samples (0.31 μg/L on August 1, 2016) and saxitoxin present in 25% of samples (3 of 12 samples).

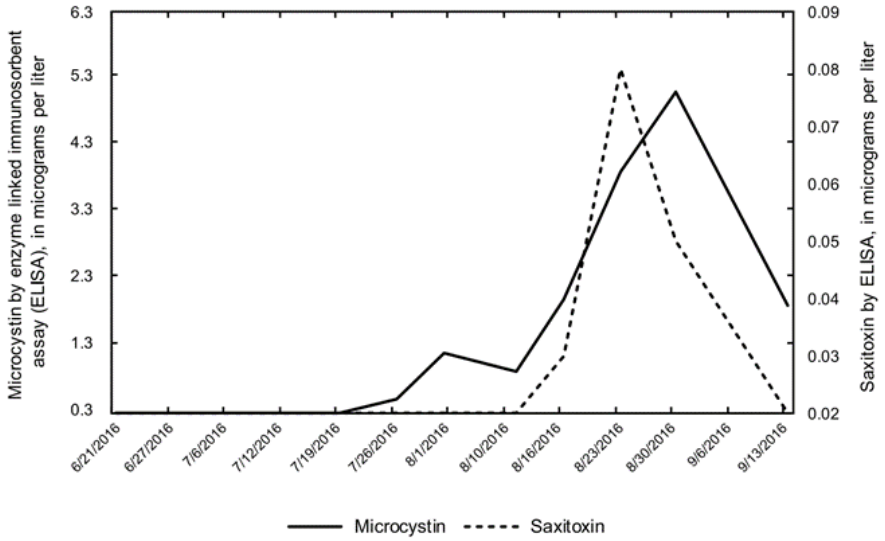

Figure 3.2. Microcystin and saxitoxin concentrations determined with enzyme-linked immunosorbent assay in water samples from Kabetogama Lake, Sullivan Bay, Northwest, near Ash River, Minnesota (U.S. Geological Survey site number 482542092493701), 2016.

3.3.4. Discussion of Changes among Phytoplankton, Cyanobacterial Genes, and Toxins

Temporal changes in the water column often affect phytoplankton dynamics (Becker et al., 2010). For example, light and nutrient availability are often correlated with increased phytoplankton growth and temperature increases can result in a decrease in green algae (Becker et al., 2010), which may allow cyanobacteria to dominate in late summer. Cyanobacterial species presence, in turn, determines which toxins can be produced; although not all strains of a particular species are capable of producing toxins (Sipari et al., 2010). In this study, total

phytoplankton biovolume peaked prior to the peak in cyanobacterial biovolume percent (Table 3.3), which is consistent with an accepted pattern of phytoplankton succession in northern temperate lakes (Wetzel, 2001). However, an additional pattern of a genus specific *Microcystis* (*mcyE*) peak (August 10, 2016; Table 3.4) was noted before the general *Microcystis* gene counts (August 16, 2016; Table 3.3). The *Microcystis* (*mcyE*) toxin gene counts in turn peaked prior to the peak in microcystin toxin concentrations (August 30, 2016; Fig. 3.2). These temporal differences provide insight into phytoplankton and cyanobacterial dominance throughout the open water season.

Dolichospermum, *Microcystis*, and *Planktothrix*, three toxin-producing genera, were prevalent during August; however, each genus also had a small peak in mid-July (Christensen and Olds, 2019). All three toxin producers were present in 10 of 12 (83%) samples collected in 2016 and, whereas microcystin is known to recur in waterbodies, anatoxin-a occurrence is less consistent through time (Sabart et al., 2015). Both saxitoxin (*sxtA*) and anatoxin (*anaC*) genes also peaked during this period, prior to field observations of serious or extreme blooms at the surface. Although toxin concentrations were relatively low early in the season (Fig. 3.2) the possibility of toxin presence early in the open water season before visible blooms are present cannot be ruled out. This reinforces the concept that one cannot simply look for the absence of a visible bloom as an indication of the absence of cyanotoxins because toxins may be present in the absence of a bloom and may have been produced by benthic taxa or by planktonic cyanobacteria that have since lysed. Conversely, the presence of a bloom does not always indicate the toxin is present, and high toxin levels can occur when *mcyE* genes are not present (Gobler et al., 2007).

Additionally, results of phytoplankton identification (Christensen and Olds, 2019) revealed the presence of *Aphanizomenon*, a known producer of anatoxin-a and saxitoxin. Because most toxin is produced in mid- to late August (Fig. 3.2), one might conclude that *Aphanizomenon* is responsible due to its abundance in mid-August (Fig. 3.1). However, because several possible toxin producing cyanobacterial species exist at once in the lake (*Dolichospermum*, *Microcystis*, *Planktothrix*, and *Aphanizomenon*), any of them has the potential to become dominant or form blooms in response to changed conditions (Rantala et al., 2006), which highlights the importance of studying the co-occurrence of multiple toxin producers. In addition, both toxic and non-toxic strains of cyanobacteria were present in blooms and it is possible that the main toxin producer is not the dominant genus in terms of cell abundance or biovolume.

Microscopic analysis of the samples also identified cf. *Cylindrospermopsis* in one sample during the current study (August 1, 2016; Christensen and Olds, 2019). *Cylindrospermopsis*, which is generally found in tropical and subtropical lakes (McCarthy et al., 2009; World Health Organization, 2003), has been reported to produce the toxin cylindrospermopsin (Briand et al., 2004). However, other researchers reported *Cylindrospermopsis* strains in the USA were genetically different from strains found in other parts of the world (Dyble et al., 2002; Gugger et al., 2005b) and lacked the capability to produce cylindrospermopsin (Yilmaz et al., 2008). Cylindrospermopsin analysis was not included in 2016, but 2017 qPCR gene assays and ELISA tests (U.S. Geological Survey, 2019) indicated its presence in samples from Kabetogama Lake.

The qPCR analysis helped clarify when toxic and non-toxic strains occurred. Although cell count information may be used as a preliminary assessment of the hazard presented by the sample (Chorus and Bartram, 1999), several misconceptions are possible if a resource manager

were one to rely on microscopic phytoplankton analysis alone to determine the cyanobacterial composition of the samples. Microscopic phytoplankton analysis without qPCR analysis cannot differentiate between toxic and non-toxic strains of these genera. Due to the lack of differentiation, one could mistakenly conclude that a bloom was capable of producing toxins when it was not, based on a microscopic analysis with high numbers of cyanobacteria present. Moreover, cyanobacteria cell abundance often is used as a surrogate for microcystin toxin values (Loftin et al., 2016), and if cell abundance were compared to microcystin concentrations, most samples from Kabetogama would be in the moderate risk category. The use of cell counts to estimate hazards can overestimate the risk to recreational users (Farrer et al., 2015). Therefore, the use of the qPCR technique for this study was valuable for differentiating whether the bloom was capable of producing a toxin.

When algal biovolume data and general cyanobacterial gene abundance were compared, which included toxic and non-toxic strains (Fig. 3.1), general cyanobacterial gene abundance peaked prior to the peak in algal biovolume (August 16 versus August 23). This may be an indication that smaller cyanobacteria are present during the earlier peak. *Dolichospermum* microcystin toxin genes (*mcyE*) had a slight peak (August 1, 2016; Fig. 3.3a) prior to the general *Dolichospermum* genes (August 10; Fig. 3.3a). Results for *Microcystis*, which produces microcystin, had a similar pattern with the maximum *Microcystis* microcystin toxin genes (*mcyE*) occurring the week prior to the general genes (August 10 versus August 16; Fig. 3.3b). This has important implications for public health, because warnings are of little use when they come after the peak in toxin production.

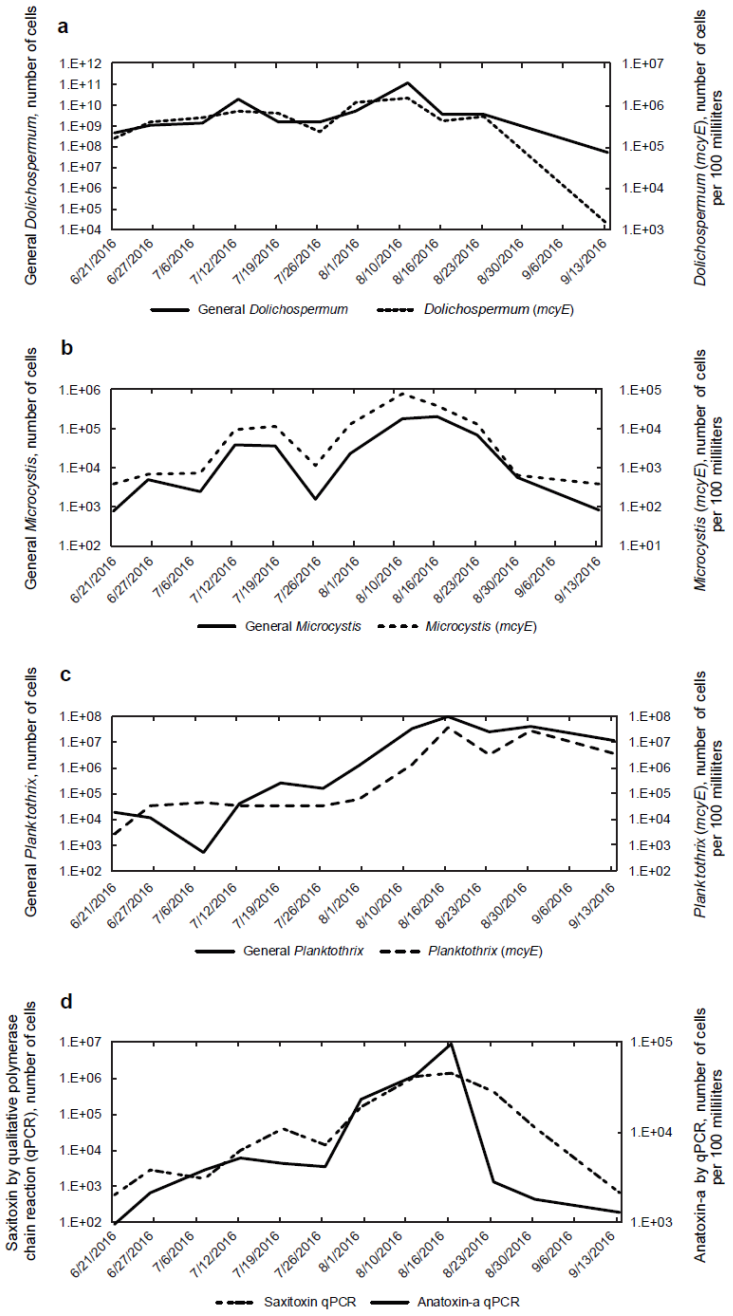

Figure 3.3. Graphs of (A) *Dolichospermum* and *Dolichospermum* (*mcyE*), (B) General *Microcystis* and *Microcystis* (*mcyE*), (C) general *Planktothrix* and *Planktothrix* (*mcyE*), and (D) anatoxin-a and saxitoxin determined with qualitative polymerase chain reaction, Kabetogama Lake in Sullivan Bay, near Ash River, Minnesota (U.S. Geological Survey site number 482542092493701), 2016.

Anatoxin-a toxin genes (*anaC*) and saxitoxin toxin genes (*sxtA*; Fig. 3.3d) peaked after general *Dolichospermum* (*mcyE*) genes and *Microcystis* (*mcyE*) genes (August 10, Figs. 3.3a and

3b) and at about the same time as general *Planktothrix* (*mcyE*) genes (Fig. 3.3c). However, the toxin gene counts by qPCR (Fig. 3.3d) merely indicate the potential for toxin production and therefore toxin concentrations were determined to understand when toxins were produced. Maximum saxitoxin concentrations occurred on August 23, 2016 (Fig. 3.2), whereas the maximum microcystin concentration occurred a week later (August 30, 2016). The timing of toxin release has important implications:

- If water resource managers only sample for microcystin, a common practice in this region, they might miss the presence of the other toxins. For example, maximum saxitoxin concentration in Sullivan Bay occurred a week earlier than microcystin.
- Early peaks in microcystin toxin genes (*mcyE*) may be an indication of future toxin production, when conditions (perhaps higher temperatures in combination with other environmental factors) trigger the production.

The traits of certain cyanobacteria that allow them a competitive advantage over other cyanobacteria or algae may play a role in the succession of phytoplankton species in Kabetogama Lake. *Planktothrix* can withstand the continuous mixing that is common in shallow environments like Kabetogama Lake. The lakes at Voyageurs National Park are quite large, with Kabetogama Lake covering over 10,000 hectares. Therefore, wind can have a substantial mixing effect on these lakes, altering conditions such as light penetration, which can lead to fluctuating irradiance and this in turn can lead to the constant change in the dominance of different cyanobacteria. In addition, the tea-stained color of the lakes in this region (Kallemeyn et al., 2003) may reduce light penetration, and cyanobacteria have lower light requirements than green algae (Oliver and Ganf, 2000). With 52% of 2016 Secchi depth measurements below 1 m (U.S. Geological Survey, 2019) light availability may play an important role in the dominance of

phytoplankton species. *Aphanizomenon*, in particular, are adapted to relatively low light conditions (De Nobel et al., 1998).

Although urban and agricultural areas make up less than 1% of Kabetogama Lake's watershed (U.S. Geological Survey, 2011), and typical urban and agricultural nutrient sources are absent, the nutrient source may be less important than the actual nutrient concentrations in the water. Nutrient enrichment from internal loading (Christensen et al., 2013), perhaps a remnant from the logging industry (Christensen and LaBounty, 2018) or as a result of damming (Serieyssol et al., 2009), may play an important role in the excess nutrients and thus the recurring algal blooms in Kabetogama Lake. Cyanobacterial blooms can be associated with poorly flushed water (Gobler et al., 2007) and research on sediment cores from Kabetogama Lake indicated that trophic state and cyanobacteria species that thrive at higher phosphorus concentrations increased since dams were constructed (Kling, 2005). Total phosphorus concentrations at Kabetogama Lake during this study were between 0.028 and 0.074 mg/L (U.S. Geological Survey, 2019). However, similar land use and environmental setting has not led to excess nutrients at other park lakes (Kallemeyn et al., 2003). Therefore, a combination of shallow depths, continual mixing, and high nutrient drainage from wetlands upstream from Kabetogama Lake (Kallemeyn et al., 2003) may lead to conditions suitable for certain cyanobacterial taxa that can access pools of phosphorus in bottom sediments (Cottingham et al., 2015). Likely, a complex interplay between atmospheric conditions, hydrology, water chemistry, and the aquatic community affects temporal phytoplankton variability in Kabetogama Lake and the formation of cyanobacterial blooms.

As with any study, there are limitations on the interpretation of the data. The ELISA technique, although relatively easy and inexpensive to use, is not as rigorous as liquid chromatography/mass spectrophotometry, and data interpretation can be difficult (Graham et al.,

2008). Gene counts, at the genus specific or non-specific toxin level, may not be related one-to-one for the organisms that contain those genes. Some research has shown that *mcyE* gene abundance correlated to microcystin production in only a few cases (Beversdorf et al., 2015), and correlation between anatoxin or saxitoxin and *mcyE* gene abundance is not expected. Moreover, mixtures of different species of the same genus may not have the same gene copy number to cell ratio, which adds complexity to interpreting these data. Lee et al. (2015a) reports that discrepancies between results obtained with qPCR or other molecular methods and microscopy may be due to differences in preservation, counting, or amplification of other species. Finally, toxin production is both spatially and temporally variable (Huisman et al., 2018), and this report represents only 1 year of data.

3.4. Summary

Cyanobacterial blooms tend to occur repeatedly in the same water supply (World Health Organization, 2003) and the long-term consequences of repeated exposure to cyanobacteria or cyanotoxins are unknown. This is an important issue locally, where a number of lakes have experienced cyanobacterial blooms resulting in dog deaths (Heiskary et al., 2014; Lindon and Heiskary, 2009), and regionally, where both dog deaths and human illness have been reported (Trevino-Garrison et al., 2015). Microcystin has been well-documented throughout the region (Graham et al., 2010; Lindon and Heiskary, 2009); however, anatoxin-a and saxitoxin are understudied in freshwater, particularly in the north central USA (Svirčev et al., 2019).

The research presented here addressed the objectives to better identify toxin producing cyanobacteria with molecular tools (qPCR) and to better understand changes in toxin production and cyanobacterial community structure throughout an open water season. The results have

furthered the understanding of cyanobacterial blooms and toxin production in remote locations, which may lead to more informed and focused remediation strategies.

Algal blooms have been blamed on excess nutrients from both urban and agricultural sources. The uniqueness of this study is the absence of urban and agricultural sources, its remote location, and the collection of samples throughout an open water season. Previous samples at this site were collected only in late summer or early fall when algal blooms were visible.

Through DNA analysis, it was discovered that toxin-forming cyanobacteria were present before visible blooms and two toxins not previously detected in this region (anatoxin-a and saxitoxin) were present, making this study an important step to assess risk for ecological systems and human health. Early sampling of phytoplankton and qPCR analysis may be used as an advance warning to help resource managers decide quickly whether, when, and where to apply more rigorous or costly analytical methods.

CHAPTER 4: CYANOTOXIN MIXTURE MODELS: RELATING ENVIRONMENTAL VARIABLES AND TOXIN CO-OCCURRENCE TO HUMAN EXPOSURE RISK[3]

4.1. Introduction

The toxins produced by cyanobacteria (cyanotoxins) can adversely affect humans, animals, and ecosystems. The specific effects of cyanotoxins depend on the amount and the route of exposure (Trevino-Garrison et al., 2015), in addition to the type of cyanotoxin. Dermatoxins, hepatotoxins, and neurotoxins affect the skin, liver, and central nervous system, respectively (Chorus and Bartram, 1999), and are a particular concern in lakes that have recurring cyanobacterial blooms.

Most studies focus on a single toxin (microcystin; Sabart et al., 2015), yet co-existence of more than one toxin is common in freshwater bodies (Freitas et al., 2014). Cyanotoxin mixtures in aquatic environments have not been well-characterized (Graham et al., 2010) and these mixtures could have antagonistic and synergistic effects on organisms (Sabart et al., 2015). Laboratory studies have indicated a synergistic effect where a mixture of hepatotoxins and neurotoxins resulted in more severe sub-lethal effects than were indicated based on the sum of individual toxin exposures in plankton (*Daphnia magna*; Freitas et al., 2014) and mammals (Fitzgeorge et al., 1994). Human exposure to multiple toxins may result in similar synergistic

[3] The material in this chapter was co-authored by Victoria Christensen, Erin Stelzer, Barbara Eikenberry, Hayley Olds, Jaime LeDuc, Ryan Maki, Alisha Saley, Dr. Jack Norland, and Dr. Eakalak Khan. Victoria Christensen had primary responsibility for the writing the draft, data analysis, building and validating the models, and all revisions of this chapter. Erin Stelzer provided the laboratory analysis, guidance on model development, and proofreading. Barbara Eikenberry and Hayley Olds assisted with review of other Virtual Beach models and quality assurance/quality control. Jaime LeDuc, Ryan Maki, and Alisha Saley assisted with sample collection and reviewed the manuscript. Victoria Christensen is the primary developer of the model, statistics, and conclusions that are advanced here, and Drs. Jack Norland and Eakalak Khan proofread and served as advisors.

effects. Therefore, cyanotoxin mixtures may be of particular concern when evaluating human health risk and the focus on the co-occurrence of cyanotoxins fills a substantial research gap.

Although cyanobacterial blooms in freshwater systems have been increasing (Hilborn et al., 2014) and tend to occur repeatedly in the same water supply (World Health Organization, 2003), the long-term consequences of repeated exposure to cyanobacterial blooms and cyanotoxins remain unknown. Numerous studies have reported on human illness and animal deaths (Trevino-Garrison et al., 2015), however, questions remain about how to accurately assess acute and long-term risk. Therefore, the use of advanced analytical methods along with information about environmental and water-quality factors related to toxins may lead to better understanding of the triggers for toxin production in cyanobacterial populations. The results may be informative to resource managers in designing better strategies for prediction, prevention, and mitigation of cyanobacterial blooms and cyanotoxin occurrence.

The overall purpose of this portion of the dissertation research was to uncover the drivers of bloom toxicity using environmental and water-quality factors while emphasizing the necessity of collecting and analyzing samples for co-occurring toxins. Specific objectives of this study are to:

1. Identify toxin producing cyanobacteria with molecular tools;
2. Determine the relations between toxin production and selected environmental and water-quality factors to help support predictive capabilities for cyanotoxins using U.S. Environmental Protection Agency's (EPA) Virtual Beach software; and
3. Compare cyanotoxin mixture models to single toxin models.

4.1.1. Background on the Production of Cyanotoxins

Microcystins and anatoxins are produced by numerous cyanobacteria, including *Microcystis*, *Planktothrix*, and *Dolichospermum* (Bruno et al., 2017; Rantala et al., 2006) and saxitoxins are produced by these species in addition to *Lyngbya* and *Cylindrospermopsis* (Mesquita et al., 2019; Smith et al., 2019). Different phytoplankton and cyanobacteria can dominate at different times of the year (Wetzel, 2001) and cyanobacteria and cyanotoxins can co-exist within a bloom (Christensen et al., 2019). Therefore, multiple cyanobacterial species could be responsible for the toxin production at any one time, supporting strategies to sample for multiple toxins.

Optical sensors that measure algal pigments such as chlorophyll and phycocyanin, a pigment found in cyanobacteria, have been used to provide early warnings of cyanobacterial presence in recreational waters (Marion et al., 2012) and drinking-water sources (Brient et al., 2008; Izydorczyk et al., 2005; McQuaid et al., 2011). In a study of lakes in Wisconsin, precipitation, day of the year, stability, and winds were related to the presence of phycocyanin (Stone et al., 2012). The results from previous studies can be used as a starting point to determine which environmental factors may trigger cyanobacterial blooms. However, the triggers for producing cyanobacterial blooms are not necessarily the same triggers for producing cyanotoxins and it is possible that a combination of factors are involved in toxin production.

Microcystin production only can occur when the microcystin synthetase (*mcy*) gene exists in the genome (Sipari et al., 2010). Some cyanobacteria include both toxic strains with *mcy* genes and nontoxic strains without *mcy* genes (Rantala et al., 2006), which can be differentiated by molecular detection methods, such as quantitative polymerase chain reaction (qPCR; Francy et al., 2015; Sabart et al., 2015). Similarly, anatoxin production can only occur if anatoxin-a

toxin genes (*anaC*) are present in the genome, and saxitoxin production can only occur if saxitoxin toxin genes (*sxtA*) are present in the genome (Al-Tebrineh et al., 2012; Sabart et al., 2015). Therefore, determining whether these genes are present is an important component of a research study to better understand the triggers of toxin production.

4.1.2. Area of Study

Voyageurs National Park was established to commemorate French-Canadian fur traders, known as voyageurs, and to protect the scenery, geology, and waterways which comprised a part of their historic route (Public Law 91-661). The surrounding area has a continental climate with an annual average temperature of 2.9 ^{o}C (Christensen et al., 2013), short, moderately warm summers and long, cold winters. Lakes within Voyageurs National Park typically are ice covered for about 5 or 6 months per year, with ice-up occurring in mid- to late November and ice-out occurring around April 28 (Christensen et al., 2013). The frost-free season ranges from 110 to 130 days (Kallemeyn et al., 2003). Average snowfall for the nearby International Falls climate station is 172 cm and total annual precipitation is 61 cm (Christensen et al., 2013).

The park is water-based, encompassing many small lakes and part or all of four larger lakes, including Kabetogama Lake (Fig. 4.1). Morphologically, Kabetogama Lake is typical of lakes located on rocks of the Precambrian Shield. It has irregular shorelines with numerous rock outcrops and islands. Littoral areas with fine-grained sediments are primarily restricted to creek or river deltas, or protected embayments. As the largest of five hydrologically-connected lakes that make up Namakan Reservoir, Kabetogama Lake covers 10,425 hectares, with maximum and mean depths of 24 and 9 m, respectively (Christensen et al., 2011).

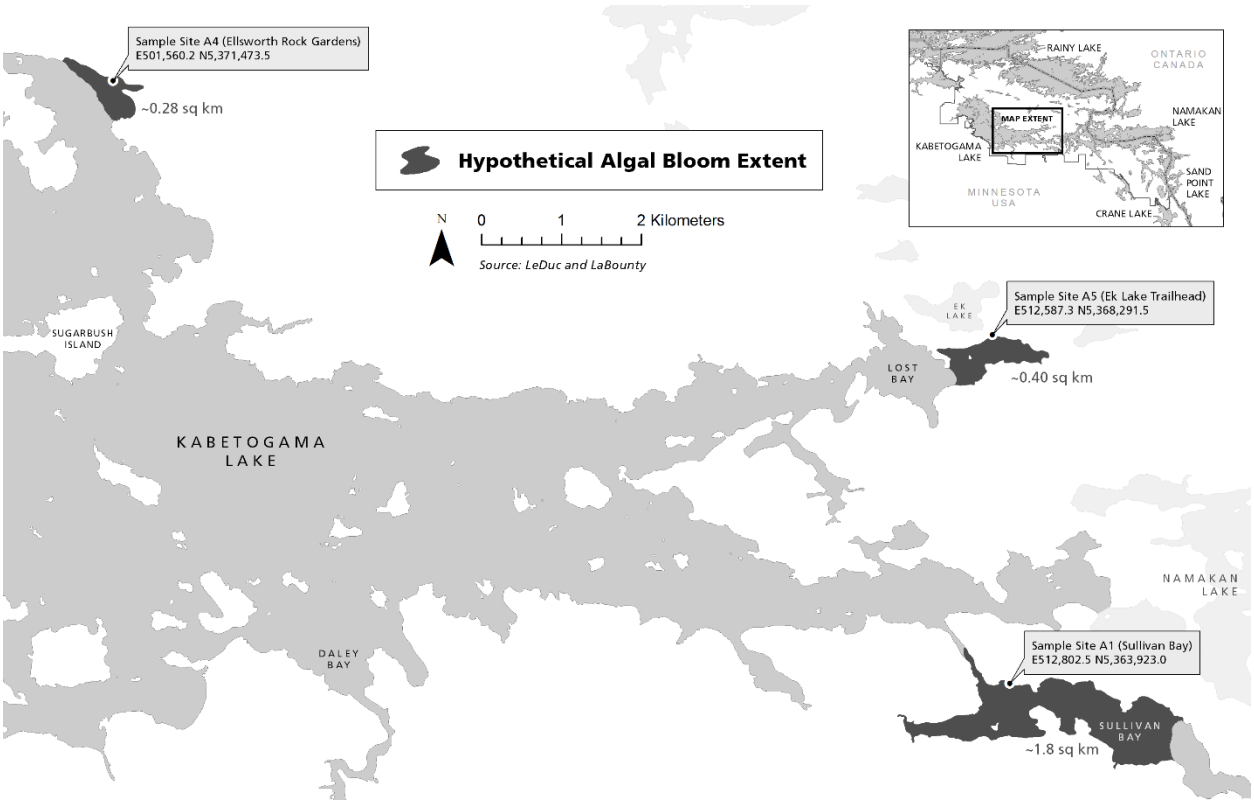

Figure 4.1. Algal bloom sites sampled in Kabetogama Lake, Voyageurs National Park, 2016-2017.

The hydrology of the system is complex, but water generally flows in a northwesterly direction. Water flows into Kabetogama Lake from several unregulated tributaries on both the north and south shores. Water flows out of Kabetogama Lake at two locations, with multidirectional flow between Kabetogama Lake and the upstream Namakan Lake (Christensen et al., 2016). Based on data from 2008, flow from Namakan Lake was the largest potential source of nutrients to Kabetogama Lake other than internal loading (the recycling of bottom sediments into the water column; Christensen et al., 2013).

The relatively shallow Kabetogama Lake has higher specific conductance (77-107 microSiemens per centimeter at 25 degrees Celsius, μs/cm) than other large lakes in the park (generally about 39-78 μs/cm; Christensen et al., 2011; Kallemeyn et al., 2003), as it receives inflow from an area that is overlain by calcareous glacial drift from the Des Moines lobe (Payne,

1991), which is generally clay rich and carbonate rich (Woodruff et al., 2002). Additionally, cottages without sewage treatment on the south side of Kabetogama Lake are a potential nutrient source to Kabetogama Lake (Payne, 1991), and the lake has polymictic circulation. These unique factors may lead to Kabetogama Lake experiencing annual cyanobacterial blooms (Christensen et al., 2013; Kallemeyn et al., 2003).

Three sites, where algal blooms recur year after year, were sampled for this study (Fig. 4.1). One site is in Sullivan Bay (USGS station 482542092493701, site A1) which lies on the south side of Kabetogama Lake where the Ash River enters Sullivan Bay before it flows into the main lake. The site is not on the main part of the lake where different water circulation patterns are likely. The site at Ellsworth Rock Garden (USGS station 482947092584401, site A4), is a popular destination for park visitors on the north shore of the main lake, adjacent to the mouth of Clyde Creek and near Cutover Island. Lastly, the site at Ek Lake Trail (USGS station 482804092494701, site A5) lies on the northeast shore of the main lake but at the end of a large bay. Therefore, all three sites are fairly distant from each other with potentially distinct water-quality characteristics.

The phytoplankton community in the cyanobacterial blooms of Kabetogama Lake includes significant biovolumes of *Microcystis* and *Dolichospermum* (formerly *Anabaena;* Christensen et al., 2019; 2011), which are known toxin-producing genera. Specifically, the cyanobacterial blooms in Kabetogama Lake frequently produce microcystin at levels exceeding the 1 μg/L drinking water guideline of the World Health Organization (WHO; World Health Organization, 2003) and at times exceeding the WHO high-risk category for recreational exposure (concentrations >20 μg/L; Chorus and Bartram, 1999; Christensen et al., 2013, 2011). Recently, two additional cyanotoxins, anatoxin-a and saxitoxin, were identified in park

waterbodies (Christensen et al., 2019). The presence of these toxins is a human health threat for shoreline residents who consume lake water and visitors to this water-based park who recreate in the water or take drinking water from the lake. Documentation of microcystin concentrations above WHO guidelines and the detection of anatoxin-a and saxitoxin, elevate the priority of the current study. Moreover, whereas models predicting microcystin exist (e.g., Nieto et al., 2011), no cyanotoxin mixture models were found in the literature.

4.2. Methodology

Intensive water sampling was needed to understand factors related to cyanobacterial blooms and cyanotoxins. Recreational locations near Sullivan Bay, Ellsworth Rock Gardens, and Ek Lake Trail were monitored whether or not blooms were present.

4.2.1. Data Collection

The three sites were sampled and environmental variables were measured once in May after ice-out, weekly in June, July, and September, and twice weekly (on subsequent days) in August. In total, 123 water samples and 12 quality-assurance samples were collected over 2 years.

Field parameters and nutrient samples were collected following procedures described by Payne (1991) and Christensen et al. (2004) for comparability to historical data. Briefly, field parameter data, including Secchi depth and water-quality monitor measurements (specific conductance, dissolved oxygen, pH, and temperature), were collected just below the water surface. Nutrient samples were collected from just below the surface with a Van-Dorn type sampler and subsamples were composited (equal volume) into 3-liter (L) bottles and placed in coolers with ice immediately after collection. Chlorophyll *a*, cyanotoxin, and cyanobacterial

gene samples were collected from about 0.3 m below the surface using dip sampling procedures (Graham et al., 2008) in wide-mouthed 1L polyethylene sample bottles.

Water samples were processed in a lakeside field laboratory where they were filtered and preserved as required. Samples for chlorophyll *a* (as described in the Quality Assurance and Quality Control section below) and nutrients were shipped from the field laboratory to the USGS National Water Quality Laboratory (Denver, Colorado) using commercial next day delivery. Cyanobacterial gene samples were filtered onto a 0.4 µm pore-size Nucleopore polycarbonate filter. The volume filtered depended on the clarity of the water, but sample volumes ranged from 25-100 mL. Each filter was placed into a 2 mL screw-capped vial with 0.3 grams of acid-washed beads and frozen. Filters were shipped to the USGS Ohio Water Microbiology Laboratory on dry ice in batches for analyses. Samples for cyanotoxins and cyanobacterial genes were stored in a freezer until batch shipment to the USGS Ohio Water Microbiology Laboratory.

4.2.2. Laboratory Analyses

Nutrient and chlorophyll analytes included: ammonia (colorimetry, salicylate-hypochlorite method), nitrite (colorimetry), nitrite plus nitrate (colorimetry by cadmium reduction-diazotization), and orthophosphate (colorimetry, phosphomolybdate method) as described by Fishman (1993); total nitrogen by alkaline persulfate digestion (Patton and Kryskalla, 2003); total phosphorus using EPA method 365.1; and chlorophyll *a* (fluorometric method; Arar and Collins, 1997).

Cyanotoxins (anatoxin-a, microcystin, and saxitoxin) were analyzed at the USGS Ohio Water Microbiology Laboratory, where lysed and filtered surface water samples were analyzed by enzyme-linked immunosorbent assays (ELISA) acquired from Abraxis, LLC (Warminster, PA) for microcystins/nodularins (United States Environmental Protection Agency, 2016),

anatoxin-a, and saxitoxins per manufacturer's directions. The minimum reporting levels (MRL) are 0.3 µg/L as microcystin-LR equivalents, 0.3 µg/L as anatoxin-a equivalents, and 0.02 µg/L as saxitoxin equivalents. Samples exceeding the highest calibration standard were diluted. ELISA analyses may represent multiple variants of each toxin (Graham et al., 2010) and, therefore, were chosen over other methods because of their ability to represent total toxins.

Cyanobacterial genes analyzed by qPCR (Stelzer et al., 2013) included: general cyanobacteria (Rinta-Kanto et al., 2005); general *Microcystis* (Rinta-Kanto et al., 2005), *Dolichospermum*/*Anabaena* (Doblin et al., 2007), and *Planktothrix*; and genus-specific *mcyE* for *Microcystis*, *Dolichospermum*/*Anabaena* (Sipari et al., 2010), and *Planktothrix* (Rantala et al., 2006); saxitoxin toxin gene (*sxtA*); and anatoxin-a toxin gene (*anaC*; Sabart et al., 2015). DNA extraction, qPCR analyses, standard curves, and quantification are described in detail in Christensen et al. (2019).

4.2.3. Quality Assurance and Quality Control

A primary quality assurance objective was to ensure that samples were representative of the cyanobacterial bloom sites in Voyageurs National Park. Quality assurance was assessed with specific procedures, such as instrument calibration, to ensure reliability and assess the quality of the sample data. The quality assurance plan for this study followed USGS guidelines and a USGS Ohio Water Microbiology Laboratory representative visited the site during the first season to provide additional training on procedures for collection and processing of samples for qPCR analysis. All field sampling equipment was maintained, calibrated, and cleaned according to the National Field Manual guidelines (U.S. Geological Survey, 2004).

Quality-control samples, consisting of replicates, blanks, and spikes were analyzed to document the variability associated with sample collection and laboratory procedures.

Effectiveness of equipment cleaning and sample processing was assessed by laboratory analysis of field blanks. Laboratory blank water was processed in the field with the same collection bottles, filtering devices, and methods as for native water samples. For cyanobacterial gene samples, one additional filter blank using sterile water was processed each day samples were filtered. Concentrations for most field blanks were below method detection limits for all constituents sampled. The two exceptions were for total phosphorus at the detection limit of 0.004 mg/L at site A1, on July 6, 2016, and for dissolved ammonia nitrogen at the detection limit of 0.01 mg/L at site A4, on August 1, 2017. These blank sample outcomes are within acceptable limits.

At the USGS Ohio Water Microbiology Laboratory, laboratory replicates and laboratory spiked matrix samples were analyzed with each batch and considered acceptable at a relative standard deviation (% RSD) equivalent to +/- 20% of average or expected value or less. More variation may be expected with field replicates due to difficulty with homogenizing intact cyanobacterial cells when splitting samples; therefore, field replicate samples were not reanalyzed if % RSD exceeded +/-20%, but rather accepted as inherent variability due to temporal or spatial variation.

For this study, within-site variability associated with sample collection and analysis was determined through the collection of concurrent field replicate samples and computation of relative percentage difference (RPD) for constituent concentrations. RPDs for all replicate pairs were ≤ 20 percent, with only one sample at 20 percent (total phosphorus on July 12, 2016 at site A1). Average RPDs for all sites were below 10 percent (Table 4.1). A typical quality-control objective for precision of replicate samples is an RPD of ≤ 20 percent (Taylor, 1987). Therefore, within-site variability represented by field replicate samples was within acceptable limits.

Table 4.1. Median relative percentage differences for nutrient concentrations in field replicate samples collected at Kabetogama Lake, Voyageurs National Park, Minnesota, 2016-2017.

Constituent	Median relative percentage difference	Pairs of replicate samples
Dissolved ammonia nitrogen (mg/L)	0	5
Dissolved nitrite nitrogen (mg/L)[a]	0	5
Dissolved nitrite plus nitrate nitrogen (mg/L)	0	5
Total phosphorus (mg/L)	10	5
Dissolved orthophosphorus (mg/L)	0	5
Total nitrogen (mg/L)	5.9	5

[a] In two pairs, one value was at the detection limit (0.001) and one was below (<0.001), therefore relative percent difference was not computed.

4.2.4. Data Analysis—Model Overview

Some preliminary statistics were completed with PC-ORD 7 software (Wild Blueberry Media, LLC, Corvallis, OR) in order to assess the potentially distinct water-quality characteristics of the three algal bloom sites within Kabetogama Lake. Analysis using non-metric multidimensional scaling and multiple response permutation procedures for all variables that were site specific (e.g., specific conductance, water temperature, toxin concentrations) did not show significant differences between sites, supporting a decision to combine sites for the model.

Subsequently, the toxin concentrations and selected environmental and water-quality factors were entered into the U.S. Environmental Protection Agency's Virtual Beach software version 3.0.6 (Cyterski et al., 2015), where data exploration and additional statistical analyses were performed. Virtual Beach (VB) was originally designed as a decision support tool for developing site-specific statistical models to predict pathogen indicator (such as *E. coli* and *Enterococci*) concentrations at recreational beaches (Cyterski et al., 2016), and VB has been successful in modelling conditions at beaches in inland lakes (Dada and Hamilton, 2016; Francy et al., 2013a), throughout the Great Lakes (Zepp et al., 2010; Francy et al., 2013b; Mednick and

Watermolen, 2014), and at marine beaches (Zepp et al., 2010; Zhang et al., 2012; Molina et al., 2014). In addition to traditional culture-based fecal indicators, VB also has been used to model molecular-based fecal indicator bacteria analyzed by qPCR (Molina et al., 2014). With VB, models can be constructed that predict a dependent variable (i.e., fecal indicator bacteria) by using independent variables such as turbidity, water temperature, specific conductance, wave height, rainfall, streamflow, various measures of stream water quality, water currents, and wave height and direction (Cyterski et al., 2016). The software provides three analytical techniques for model development, which include multiple linear regression (MLR), partial least squares regression, and a gradient boosting machine (Cyterski et al., 2016). Gradient boosting machine and MLR were identified as best-ranked methods of 5 primary methods evaluated (gradient boosting machine, MLR, partial least squares regression, binary logistic regression, and sparse partial least squares regression) in terms of predictive performance for a study that evaluated models at seven Wisconsin beaches (Brooks et al., 2016). Out of the two best ranked methods, the MLR analytical technique was chosen, because the gradient boosting machine model will not work in cases where there are no exceedances (Cyterski et al., 2015).

Although the original use for the VB model was to predict fecal indicator bacteria concentrations at beaches, it can also be used to predict other response variables, such as cyanobacterial toxin concentrations. In previous studies, two types of models have been developed to estimate microcystin concentrations at Ohio recreational sites: (1) daily predictive models using easily- or continuously-measured water quality and environmental data that does not require a site visit for sample collection, and (2) long-term comprehensive predictive models that use samples collected and analyzed in the laboratory in addition to real-time data used in daily predictive models (Francy et al., 2015). The VB model was used to construct real-time and

comprehensive models to estimate microcystin concentrations above and below recreational criteria at inland Ohio beaches and one beach on Lake Erie (Francy et al., 2016). The best real-time model (R^2= 0.82) indicated that microcystin concentrations were significantly correlated with phycocyanin, turbidity, algae category, and wind speed, whereas the best comprehensive models (R^2= 0.81 - 0.90) found that microcystin was significantly correlated to phycocyanin, pH, *Microcystis* biovolume, and *Microcystis mcyE* copies (Francy et al., 2016). Predictive modeling of cyanotoxins is a relatively new application for VB software, and has thus far only been used to estimate microcystin concentrations. Current research is lacking the application of VB for estimating other cyanotoxins of concern, such as saxitoxin and anatoxin-a or cyanotoxin mixtures.

4.2.4.1. Threshold Criteria

Field parameters and laboratory samples were used to develop equations to predict the likelihood of exceeding a threshold value. VB predicts the probability of exceeding a threshold level, such as the recreational criteria or drinking water standard, at recreational beaches. The drinking water standard was chosen, rather than the recreational standard, for the models at Voyageurs National Park because it was more protective and because the water occasionally is used for drinking in remote areas of Voyageurs National Park. Because there are no national guidelines for most toxins, the output of the models was the probability of exceeding State of Ohio guidelines (being one of the only states to have such standards for all three freshwater toxins). The guidelines for the toxins presented here were in place as of January 2017 (American Water Works Association, 2016) and are as follows: anatoxin-a (20 μg/L), microcystin (1.6 μg/L), and saxitoxin (0.2 μg/L).

4.2.4.2. Dependent Variable

The dependent variable for the microcystin only model (MC model) was microcystin concentration, which was compared to the threshold value of 1.6 μg/L, based on the drinking water guidance. The dependent variable for the cyanotoxin-mixture model (MIX model) was the sum of the normalized concentrations of anatoxin-a, microcystin, and saxitoxin. This decision was based on the desire to understand exposure risk to all three toxins analyzed and not only microcystin; however, the concentration data was normalized because toxicity and thresholds are different among the three toxins. Based on Ohio's toxin guidelines, microcystin and saxitoxin data were weighted to the highest standard of 20 μg/L (anatoxin-a); microcystin concentrations were multiplied by 12.5 and saxitoxin concentrations were multiplied by 100 to give them weights equal to the anatoxin-a standard. The guidelines used were those in place as of January 2017, because they better represent the differences in toxicity among the three toxins. Ohio's cyanotoxin drinking water guidelines have since changed to 1.6 μg/L for saxitoxins (Ohio Department of Health, 2018).

4.2.4.3. Independent Variables and Data Transformation

Factors for the independent variables were those measured with a hand-held water-quality monitor, determined from laboratory analysis, or compiled from other sources of available environmental data. Other data sources were from stations in close proximity to the sample sites. Weather data (wind speed, wind direction, barometric pressure, and precipitation variables) were obtained from the Falls International Airport weather station (KINL, in International Falls, Minn.). Photosynthetically active radiation (PAR) was obtained from the Grand Rapids, MN weather station in 2016; however, due to equipment malfunction, the PAR was obtained from the Fargo, ND weather station in 2017. Lake levels were obtained from the

U.S. Geological Survey (USGS) gage at Kabetogama Lake at Gold Portage, MN (https://waterdata.usgs.gov/nwis/uv?site_no=05129290).

Some variables were lagged, averaged, estimated, or transformed. Some nutrient and gene count variables were lagged between 4 and 8 days, for circumstances where the effects on cyanobacteria and toxin production may be delayed. In accordance with previous VB (Searcy et al., 2018) and other MLR models (Carpenter et al., 2014), the use of censored data (below detection limits or "less thans") was deemed acceptable and censored data were estimated by converting censored values to half the detection limit. For qPCR values that had more than one detection limit, censored values were converted to half of the lowest detection limit. Using the VB software, some independent variables were transformed to linearize the data.

Although 123 samples were collected as part of this research effort, not every analysis could be performed on each sample and not all explanatory variables were available for all sample dates. Because missing cells are not allowed in VB and entire sampling dates had to be deleted, the resulting models were based on 50 complete samples. Independent variables with greater than 85% censored data (orthophosphorus, nitrate, and nitrite) were eliminated. For inclusion in the final models, explanatory variables were limited to a variance inflation factor (VIF, a measure of multicollinearity among variables) of 5 or less. Additionally, the maximum number of variables was limited to six and these were chosen based on Akaike's Information Criterion, corrected for small sample size (AICc). The final equation was chosen based on the predicted residual error sum of squares (PRESS). Both AICc and PRESS are available measures within the VB software. Generally, model diagnostics and selection were completed as described by Francy and Darner (2006) and Francy et al. (2016).

Some environmental variables were summed or averaged for a variety of antecedent periods. Rainfall was summed for the previous 14 days. Lake level change (in feet) over the previous 24 hours underwent two calculations. For the first, the lake level change at 10 a.m. each day was averaged over 7 days. For the second, the absolute value of the lake level change at 10 a.m. each day was averaged over 7 days. The 24-hour sum and a 3-day sum were calculated for the PAR variable.

4.3. Results and Discussion

Summary statistics for water-quality varied little among the three sites (Table 4.2). Median N:P ratios were calculated in an effort to understand the triggers of the blooms. Orihel (2015; 2012) indicated that N:P ratios below 23 have higher microcystin concentrations. In Kabetogama, median N:P ratios ranged from 20.0 – 23.9 (Table 4.2), indicating that there is high risk for microcystin throughout most of the summer season. However, N:P ratios may be more useful to indicate risk on a regional scale (e.g. Orihel et al., 2012) and not seasonally. Moreover, and the N:P ratio preceding the sample date may be more important in influencing toxin production than the median N:P ratio or the N:P ratio at the time of sampling in Voyageurs National Park.

4.3.1. Toxin Concentrations

For anatoxin-a, site A1 was the only site with concentrations above the detection limit (0.30 μg/L; Table 4.3). Although Ohio's cyanotoxin guidelines were used for the models, Voyageurs National Park is within the state of Minnesota, which has health-based guidance (for short term exposure of between 1 and 30 days) for both anatoxin-a and microcystin. All anatoxin-a detections greater than the detection limit exceeded the state of Minnesota's anatoxin-a health-based guidance of 0.1 μg/L (Minnesota Department of Health, 2016). Microcystin

concentrations ranged from <0.30 to 5.2 μg/L, with the highest concentrations occurring at site A5, followed by site A1. Because the limit of detection for anatoxin-a and microcystin by ELISA (0.30 μg/L) test is greater than the state of Minnesota health-based guidance (0.1 μg/L), some of the <0.30 μg/L ELISA results could have exceeded the guidelines as well. Exceedances for anatoxin-a and microcystin were not unexpected, due to the selection of these sites at recurring bloom locations and sample collection during active blooms. Saxitoxin concentrations ranged from <0.1 to 0.13 μg/L, with detections at sites A1 and A5. No saxitoxin guidance is currently (2020) available for the state of Minnesota or at a National level; however, no sample exceeded the state of Ohio saxitoxin guidelines of 0.2 μg/L (2017) or 1.6 μg/L (2020).

Table 4.2. Summary statistics for water-quality measurements in Kabetogama Lake, Voyageurs National Park, 2016-2017 (June through October).

Year	Metric	WT	D	SC	DO	pH	NH_3	N+N	OP	TP	TN	PC	N:P
U.S. Geological Survey site number 482542092493701, site A1													
	Minimum	18.2	0.1	93	4.5	7.1	< 0.01	<0.040	< 0.012	0.026	0.64	1.4	
2016	Maximum	25.6	1.6	205	17.6	9.1	0.03	0.041	0.025	0.074	1.49	3.6	
	Median	22.3	0.9	189	9.9	8.3	< 0.01	<0.040	< 0.012	0.052	1.13	8.0	21.7
	Minimum	15.9	0.8	100	7.5	7.3	< 0.01	< 0.040	< 0.012	0.010	0.32	1.7	
2017	Maximum	25.2	2.3	229	13.0	8.9	0.01	< 0.040	< 0.012	0.045	0.94	2.3	
	Median	21.4	1.15	202	10.0	8.3	< 0.01	NC	NC	0.031	0.68	2.0	21.8
U.S. Geological Survey site number 482947092584401, site A4													
	Minimum	17.7	1.0	88	5.0	7.5	< 0.01	< 0.040	< 0.012	0.015	0.38	0.5	
2016	Maximum	24.1	2.2	97	15.7	9.0	0.08	< 0.040	< 0.012	0.056	0.89	8.0	
	Median	21.9	1.9	93	9.1	8.1	< 0.01	NC	NC	0.028	0.56	2.1	20.0
	Minimum	16.2	1.0	100	7.5	6.2	< 0.01	< 0.040	< 0.012	0.007	0.29	1.1	
2017	Maximum	25.4	2.1	165	12.3	8.7	0.060	0.060	0.057	0.061	0.74	1.3	
	Median	20.9	1.5	103	9.1	7.6	NC	NC	NC	0.021	0.49	1.2	23.9
U.S. Geological Survey site number 482804092494701, site A5													
	Minimum	16.9	0.9	88	1	7.6	< 0.01	< 0.040	< 0.012	0.017	0.42	1.0	
2016	Maximum	27.5	2.7	97	15.2	9.3	0.03	< 0.040	< 0.012	0.069	1.36	6.7	
	Median	24.2	1.75	92	9.6	8.8	< 0.01	NC	NC	0.026	0.60	2.8	23.1
	Minimum	16.6	0.8	98	7.9	6.9	< 0.01	< 0.040	< 0.012	0.008	0.28	0.7	
2017	Maximum	27.0	3.8	199	13.0	8.9	0.03	0.104	0.168	0.038	1.05	0.7	
	Median	22.0	1.9	102	9.4	8.2	< 0.01	NC	NC	0.025	0.53	0.7	21.2

[1]The phycocyanin sensor malfunctioned in 2017, with only 1 (site A5) or 2 valid measurements (sites A1, A4) for 2017 statistics.

Table 4.3. Summary statistics for anatoxin-a, saxitoxin, and microcystin by enzyme-linked immunosorbent assay (ELISA) in Kabetogama Lake, Voyageurs National Park, 2016-2017 (June through October). All measurements in micrograms per liter.

Site	Year	Metric	Anatoxin-a	Microcystin	Saxitoxin
A1	2016	Minimum	<0.30	<0.30	<0.02
		Maximum	0.31	5.1	0.08
		Median	0.305	0.9	<0.02
	2017	Minimum	<0.30	<0.30	<0.02
		Maximum	0.61	0.32	0.07
		Median	0.34	<0.30	0.03
A4	2016	Minimum	<0.30	<0.30	<0.02
		Maximum	<0.30	0.94	<0.02
		Median	<0.30	<0.30	<0.02
	2017	Minimum	<0.30	<0.30	<0.02
		Maximum	<0.30	0.52	<0.02
		Median	<0.30	<0.30	<0.02
A5	2016	Minimum	<0.30	<0.30	<0.02
		Maximum	<0.30	0.46	0.09
		Median	<0.30	<0.30	<0.02
	2017	Minimum	<0.30	<0.30	<0.02
		Maximum	<0.30	<0.30	0.13
		Median	<0.30	<0.30	0.05

4.3.2. Relations between Comprehensive Variables, Environmental Conditions, and Toxin Concentrations

One of the goals of the VB model is to be able to predict toxin concentrations in real time to use as a guide to inform advisory decisions. The alternate is collecting water samples and sending them to a laboratory for analysis, resulting in water managers receiving information when it is too late to issue an advisory. However, the goal for this research was to determine whether a toxin mixture was a useful measure of exposure risk compared to a single toxin and if different environmental triggers would be identified for the MIX model versus the MC model. Therefore, explanatory variables that were available in real time, as well as laboratory analyses,

were considered. The environmental variables considered for inclusion and uploaded into VB were those that were known to influence cyanobacteria or their toxins based on previously published studies. The most important consideration was theoretical relevance; each individual variable was plausible as an influence on toxin concentrations. For example, temperature, pH, and PAR influenced anatoxin-a in laboratory experiments (Kaminski et al., 2013) and phosphorus was suggested as a limiting nutrient for plankton (including toxin-producing cyanobacteria) in nearby Lake of the Woods (Watson and Kling, 2017). Therefore, the upload of variables to the VB software included field variables, environmental or weather variables, laboratory variables, and time of year as described in Methodology.

Eleven model variables (Table 4.4) were significant for at least one of the final VB equations. The goal of multiple linear regression is to explain as much of the variation in the dependent variable (weighted-toxin concentration or microcystin concentration) as possible, and independent variables can be included in an equation whether or not the individual variable is significant (Helsel and Hirsch, 1993). For consistency, both MC and MIX models used the same set of possible independent variables, with the exception that variables only relevant to anatoxin-a or saxitoxin were not used in the MC model.

Table 4.4. Parameters used as independent variables in multiple linear regression equations developed for algal bloom sites in Kabetogama Lake, Voyageurs National Park.

Variable	Key
Measured at bloom sites (measured variables)	
Water temperature (degrees Celsius)	WT
Specific conductance (μS/cm)	SC
Total phosphorus (mg/L as P)[1]	TP
Planktothrix-specific microcystin toxin gene - *mcyE* (copies/100 mL) by qPCR[1]	Plank_mcyE
Microcystis-specific microcystin toxin gene - *mcyE* (copies/100 mL) by qPCR[1]	Micro_mcyE
Anatoxin-a toxin gene - *anaC* (copies/100 mL) by qPCR[1]	anaC*
Saxitoxin toxin gene - *sxtA* (copies/100 mL) by qPCR[1]	sxtA*
Measured at nearby stations (environmental variables)	
Wind direction, instantaneous, in degrees	WindDir
Lake level change in feet over the last 7 days at 10 a.m. (including sampling day)	LL_7day
The difference between lake level on the day of sampling and the spring average (April and May) lake level, in feet	LL_Spring
Lake level change in feet over the last 14 days at 10 a.m. (including sampling day)	LL_14day

*variables specific to anatoxin-a or saxitoxin were not used in the microcystin (MC) model; [1]variables were lagged between 4 and 8 days.

4.3.3. Comprehensive Models for Estimating Microcystin and Weighted-toxin Concentrations in Kabetogama Lake

Initial modeling efforts included all possible variables listed in Methodology (comprehensive models). The best comprehensive MC and MIX models based on PRESS (Table 4.5) contained wind direction and lake level parameters. The best comprehensive MC model also included qPCR-derived parameters (MC_mcyE, Plank_mcyE), whereas the best comprehensive MIX model included total phosphorus as well as qPCR-derived parameters (Plank_mcyE, anaC, and sxtA). The coefficient of determination (R^2) of the MC and MIX models were unexpectedly close at 0.87 and 0.86, respectively, indicating that the goodness of fit is similar between the models.

Table 4.5. Best comprehensive equations from the Virtual Beach software for microcystin concentration and weighted-toxin concentrations in Kabetogama Lake, 2016-2017.

MC = 0.7327 – 34.67*INVERSE[WT] + 344.4*SQUARE[TP] + 0.0020*SQUAREROOT[MC_mcyE] + 0.0444*QUADROOT[Plank_mcyE] + 0.0010*WindDir + 9.013*LL_7day

Model	AICc	PRESS	R^2	Sensitivity	Specificity
Comprehensive MC	-15.69	14.92	0.87	0.88	0.98

MIX = -12.3423 + 4,593*SQUARE[TP] + 0.4540*QUADROOT[Plank_mcyE] + 2,672*INVERSE[anaC] + 0.0167*SQUAREROOT[sxtA] + 0.8216*WindDir – 0.0212*INVERSE[LL_14day]

Model	AICc	PRESS	R^2	Sensitivity	Specificity
Comprehensive MIX	.0244	.00246	0.86	1.00	0.95

One important consideration for modeling at beaches or recreation areas is the number of false positives and false negatives generated from the model. For the models, the decision threshold was the same as the regulatory guideline. The comprehensive MC model generated one false positive (Fig. 4.2a), indicating that the model incorrectly indicated an exceedance of the established threshold. Consequently, if decisions were based on this number, unnecessary restrictions, closures, or warnings might be put into place when true microcystin concentrations were below the threshold value. The model also generated one false negative (Fig. 4.2a), indicating that microcystin was present above the threshold but the comprehensive MC model did not simulate an exceedance, potentially putting recreational users at risk. This concentration was at 2 μg/L (Fig. 4.2b), which is above the drinking water standard (and thus the threshold value) of 1.6 μg/L, but below WHO's recreational standard of 20 μg/L.

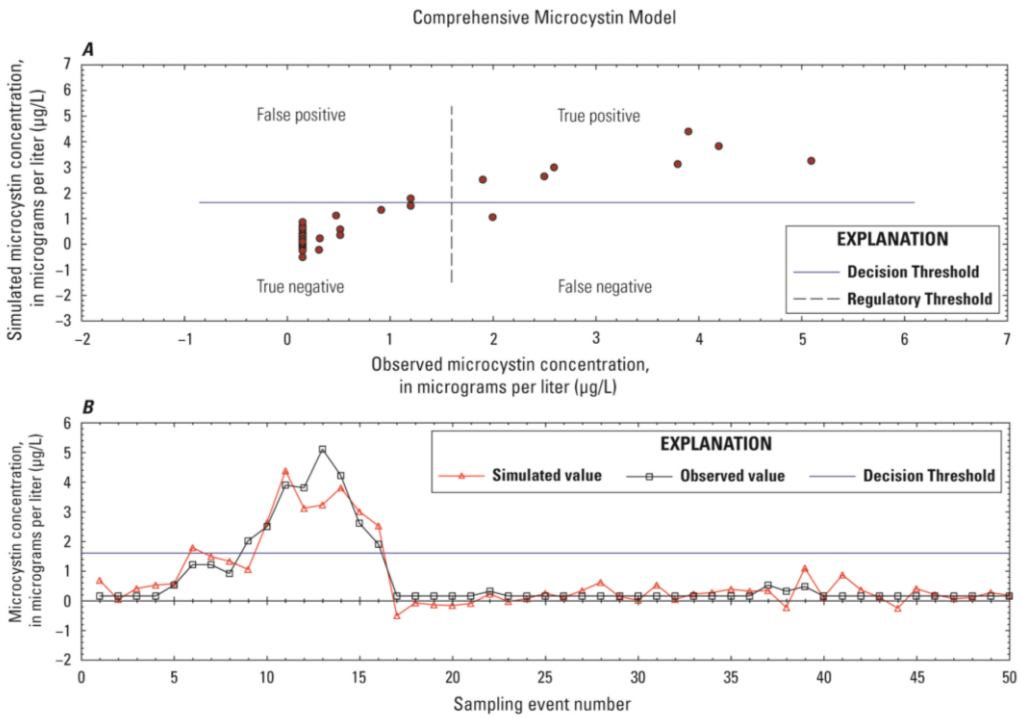

Figure 4.2. (A) False positives (model predictions exceeded the decision threshold, but observations were below the regulatory guideline) and false negatives (model predictions were below the decision threshold but observations exceeded the regulatory guideline), and (B) observed and simulated microcystin concentrations in chronological order for the selected comprehensive microcystin (MC) model for Kabetogama Lake, Voyageurs National Park, 2016-2017.

For the comprehensive MIX model there were two false positives (Fig. 4.3a) and no false negatives, although one data point was at the threshold limit of 20 µg/L between the true positive and false negative (Fig. 4.3a). In this situation a false positive may keep recreational users away unnecessarily, but without any false negatives, human health risk would be minimized since the MIX model was able to simulate every exceedance.

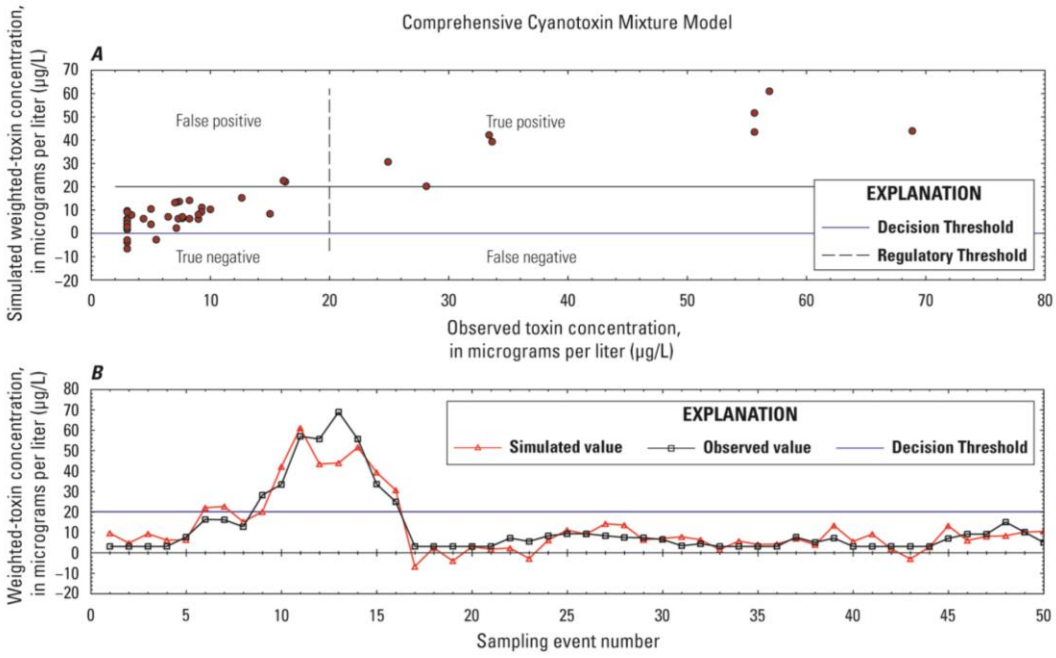

Figure 4.3. (A) False positives (model predictions exceeded the decision threshold, but observations were below the regulatory guideline) and false negatives (model predictions were below the decision threshold but observations exceeded the regulatory guideline), and (B) observed and simulated weighted-toxin concentrations in chronological order for the selected comprehensive cyanotoxin mixture (MIX) model for Kabetogama Lake, Voyageurs National Park, 2016-2017.

4.3.4. Best Environmental Models for Estimating Microcystin and Weighted-toxin Mixture Concentrations in Kabetogama Lake

Because intensive water sampling is not continuous at Kabetogama Lake, additional equations that included only readily available environmental and weather variables were modeled (Table 4.6). Both MC and MIX best fit environmental models included the same set of independent variables (SC, LL_7day, and LL_Spring). Not unexpectedly, the R^2 values for these equations are not as strong as the comprehensive models but are similar to each other at 0.58 (MC) and 0.57 (MIX).

Table 4.6. Best equations from the Virtual Beach software for microcystin concentration and weighted-toxin concentration in Kabetogama Lake, using environmental variables only, 2016-2017.

MC = -1.898 – 189.3*INVERSE[SC] + 18.58*LL_7day + 6.132*INVERSE[LL_Spring]					
Model	**AICc**	**PRESS**	**R^2**	**Sensitivity**	**Specificity**
Environmental MC	32.80	38.00	0.58	1.00	1.00
MIX = -20.60 – 2519*INVERSE[SC] + 238.6*LL_7 + 78.37*INVERSE[LL_Spring]					
Model	**AICc**	**PRESS**	**R^2**	**Sensitivity**	**Specificity**
Environmental MIX	291.9	6791	0.57	0.74	0.74

The environmental MC model had no false positives and no false negatives (Fig. 4.4a) and all values exceeding the threshold were simulated by the model (Fig. 4.4b). However, no microcystin concentration exceeded the recreational criteria of 20 μg/L (Fig. 4.4b). The environmental MIX model had 3 false positives and no false negatives (Fig. 4.5a), indicating that if decisions were made based on this model, unnecessary restrictions might be put into place when toxin concentrations were below the threshold value, however, the three false positives were near the weighted-toxin threshold of 20 μg/L (predicted values: 22.2, 20.9, and 21.1 μg/L; Fig. 4.5b) and the measured weighted-toxin concentrations were slightly below this threshold (observed values: 16.3, 16.1, and 12.6 μg/L; Fig. 4.5b). The lack of false negatives for either environmental model indicates that human risk would be at a minimum, since no exceedances were simulated below the threshold.

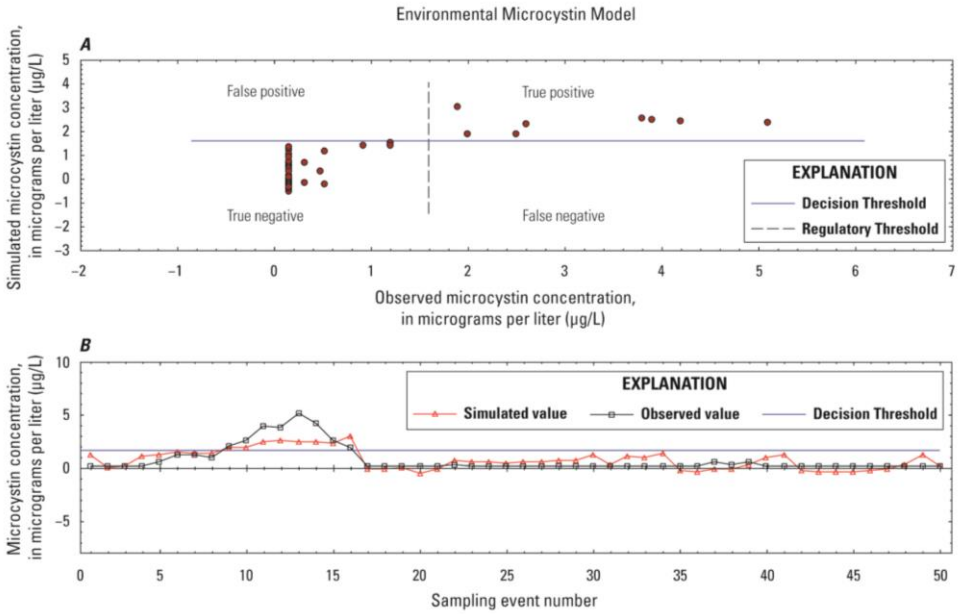

Figure 4.4. (A) False positives (model predictions exceeded the decision threshold, but observations were below the regulatory guideline) and false negatives (model predictions were below the decision threshold but observations exceeded the regulatory guideline), and (B) observed and simulated weighted-toxin concentrations in chronological order for the selected environmental microcystin model for Kabetogama Lake, Voyageurs National Park, 2016-2017.

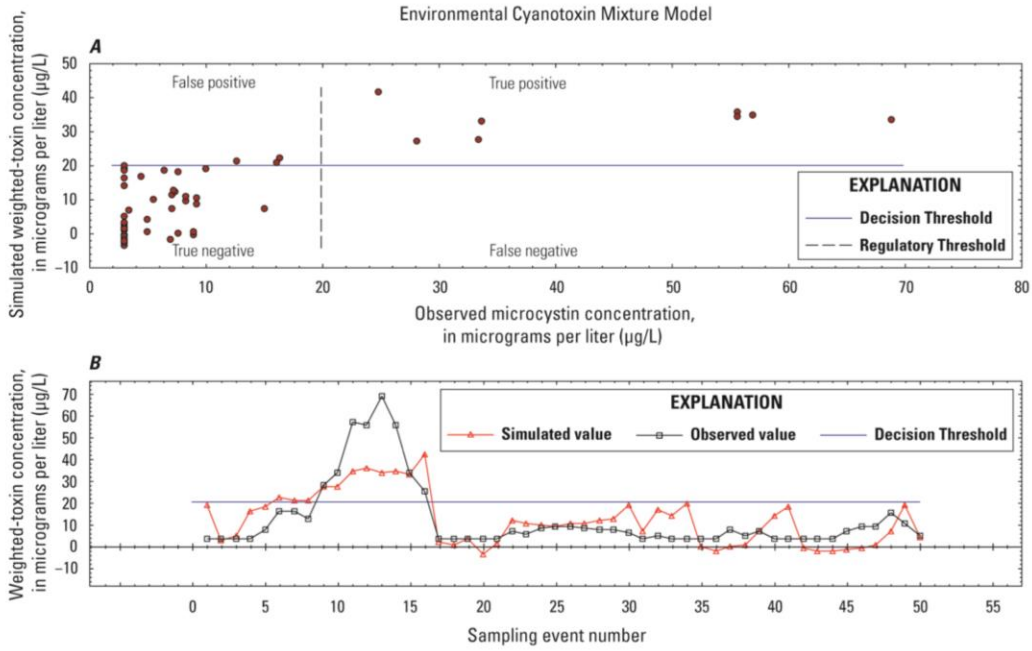

Figure 4.5. (A) False positives (model predictions exceeded the decision threshold, but observations were below the regulatory guideline) and false negatives (model predictions were below the decision threshold but observations exceeded the regulatory guideline), and (B) observed and simulated weighted-toxin concentrations in chronological order for the selected environmental cyanotoxin mixture model for Kabetogama Lake, Voyageurs National Park, 2016-2017.

4.3.5. Relations Between Microcystin Concentrations and Comprehensive and Environmental Variables

Many factors were correlated with microcystin concentration, which could potentially be used in a comprehensive or environmental model. The independent variables related to microcystin concentrations in the selected comprehensive MC model were variations of water temperature, total phosphorus, the microcystin *mcyE* gene count, *Planktothrix*-specific *mcyE* gene count, wind direction, and lake level change. Each of these parameters has some relevance related to previous research. Water temperature, for example, has shown a relation to microcystin in a VB model (Francy et al., 2015) and has been shown to regulate microcystin production in laboratory experiments (e.g., Walls et al., 2018).

Research has shown that concentrations of phosphorus and chlorophyll, Secchi depth, temperature, and other environmental factors are related to microcystin occurrence (Kotak et al., 2000). For example a study of 241 water bodies in the midwestern United States found that Secchi depth and latitude were related to microcystin concentrations (Graham et al., 2004). The influence of nutrients on cyanobacterial blooms has been studied extensively (e.g., Lapointe et al., 2015; Orihel et al., 2015). Specifically, total phosphorus has been correlated with microcystin production (Kotak et al., 1995). However, the relation between toxin production and nutrients is more complicated (Eldridge and Wood, 2019), with nutrient form (Monchamp et al., 2014) and N:P ratios (Orihel et al., 2015) adding to that complexity. A study of over 246 water bodies across Canada (Orihel et al., 2015) reported that high microcystin concentrations occur only at low N:P ratios in nutrient-rich lakes. The relative influence of phosphorus at the shallow and polymictic Kabetogama Lake might be further complicated by the frequent recycling and internal loading of phosphorus from bottom sediments (Christensen et al., 2013). Although many

dissolved nutrients were primarily below the detection level and, therefore, were not used in the final VB models, the remaining N:P data had no relation to microcystin concentrations.

Gene counts for both *Microcystis*-specific *mcyE* and *Planktothrix*-specific *mcyE* were related to microcystin concentration in the comprehensive MC model. The *mcyE* gene must be present in order for the cyanobacteria to produce microcystin (Beversdorf et al., 2015; Sipari et al., 2010), so the relation between *mcyE* gene counts and microcystin concentrations was not unexpected. Moreover, research has shown that most strains of both *Microcystis* and *Planktothrix* produce microcystins (Rantala et al., 2006) and other researchers have shown a correlation between microcystin and *mcyA* (Sabart et al., 2015) and *mcyE* gene counts (Francy et al., 2016). Conversely, Beversdorf et al. (2015) failed to show correlation between *mcyE* gene counts and microcystin concentration in three lakes. For the model the *mcyE* gene counts were lagged (the *mcy*E gene count between 4- and 8-days prior was used, depending on previous sample collection date), which may better represent the conditions leading up to toxin production and thus account for the correlation and inclusion of *mcyE* gene count in the comprehensive MC model.

The independent variables for the environmental MC model included specific conductance (SC) and variables related to lake level (LL_7day, LL_Spring). Whereas Francy et al. (2015) found no correlation between microcystin concentration and SC in Lake Erie, Lindon and Heiskary (2009) noted microcystin and SC had an inverse relation in 50 Minnesota lakes, some in close proximity to Voyageurs National Park. A major component of SC is salinity, which is a measure of the dissolved salts in water. Rosen et al. (2018) showed that increased salinity had an effect on the cyanobacterial cell membrane, causing microcystin to leak from the cells. Additionally, Rosen et al. noted some evidence of microcystin biosynthesis by *Microcystis*

aeruginosa at salinities greater than 7.5 practical salinity units. *Microcystis* was identified in samples from Voyageurs National Park during 2016-2017 (Christensen and Olds, 2019), although individual species of the genus were not identified. However, *Microcystis aeruginosa* was present during 2019 (Appendix, Table A1) and may have been present in 2016-2017. Moreover, it may be possible that other microcystin producers are affected by greater salinity. The relation between microcystin and SC in Kabetogama Lake may be due to receiving water from an area of glacial drift that is unusually rich in carbonate and soluble minerals (Payne, 1991; Woodruff et al., 2002), resulting in an SC of about 77-107 μS/cm (Christensen et al., 2016). However, the complex hydrology that includes multidirectional flow between lakes in the system with substantially lower SC (generally about 39-78 μS/cm; Kallemeyn et al., 2003) could complicate this relation in future years of unusually high or low inflow.

The lake-level variables are important because the water levels in the Voyageurs National Park lake chain have been manipulated by dams since the early 1900s to regulate water flow and provide hydropower for the timber industry (Serieyssol et al., 2009). Today, the dams continue to be managed to provide power to the pulp and paper industry, but also are managed to provide suitable conditions for game fisheries and recreation (Christensen and LaBounty, 2018). Since 2000, the management of the water levels has been balanced for the benefit of many interests, including ecological and environmental values (International Rainy Lake Board of Control, 1999).

Two different lake level variables were included in the model. The first was the average lake-level change over the previous 7 days. Streamflow, or discharge, was related to microcystin concentration in VB models for Lake Erie and inland Ohio lakes (Francy et al., 2015), including a variable for 7-day average antecedent discharge. Because many of the inflows to Kabetogama

Lake are not measured, lake level was deemed the best variable to capture these changes, as it is related to both the inflows and outflows of the system; the antecedent lake level is important because it is related to residence time and long residence times can lead to bloom formation (Merel et al., 2013). The residence time for Namakan Reservoir (which includes Kabetogama Lake) was reported as 235 days by (Kallemeyn et al., 2003). Residence times for Kabetogama Lake have not been determined, but typically lakes with less volume, like Kabetogama Lake, would have a shorter residence time. However, Kabetogama Lake has two outlets and multidirectional flow (Christensen et al., 2016) and may act like a backwater area to the larger Namakan Lake (Christensen et al., 2011); backwater areas are subject to cyanobacterial proliferation (Grace et al., 2010; Rosen et al., 2018).

The second water level variable that was related to microcystin concentration in the VB model was the difference in lake level elevation on the day of sampling from the spring average (LL_ Spring). This variable may be related to the inundation of shallow wetland areas and how often they are hydrologically connected to Kabetogama Lake. These wetlands also may be considered backwater areas and may serve as sources of nutrients to Kabetogama Lake, and frequent inundation of these areas may lead to more nutrients in the recurring bloom areas within the lake. The relation between microcystin concentration and this lake level variable is inverse, and one possibility is that falling water levels indicate more stagnant water conditions. Stagnant conditions may result in buoyant cyanobacteria moving closer to the surface to form blooms (Huisman et al., 2018; Rastogi et al., 2015), ultimately exposing them to light (Huisman et al., 2018), which may trigger toxin formation or release.

4.3.6. Relations Between Weighted-toxin Concentrations and Comprehensive and Environmental Variables

The weighted-toxin concentrations are composed of anatoxin-a and saxitoxin, as well as microcystin, therefore, due to the different modalities of neurotoxins and hepatotoxins, it is plausible that different factors affect both the comprehensive and environmental models. For the comprehensive MIX model, total phosphorus, gene count, and lake level variables were included in the equation, similar to the MC models except the that the lake level variable was the inverse of the 14-day lake level change rather than the 7-day. There may be a physical or functional reason for the difference in relation to a 7-day lake level change or a 14-day lake level change, but the difference in these two measurements over the course of the study was very small (-0.025 and -0.028 ft, respectively).

In addition to lake levels, both *anaC* gene counts and *sxtA* gene counts were included in the comprehensive MIX model. However, *anaC* had an inverse relation to the weighted-toxin concentration. On the surface, an inverse relation between a gene count and toxin concentration may not seem likely, but anatoxin-a only occurred at site A1 and its maximum concentration occurred in 2017, whereas maximum microcystin concentration occurred in 2016. Moreover, anatoxin-a and microcystin did not peak at the same time in 2016 (Christensen et al., 2019). It is plausible that microcystin is produced primarily when anatoxin-a is not and, therefore, microcystin concentrations may be high when *anaC* genes are low, perhaps due to competition among microcystin- and anatoxin-producing species of cyanobacteria. Salmaso et al. (2016) noted that most samples with isolated strains of cyanobacteria containing *anaC* produced anatoxin-a, however in a study by Sabart et al. (2015) *anaC* and anatoxin-a did not correlate. Similarly, Savela et al. (2017) found no correlation between *sxt*A gene count and saxitoxin,

although most samples showed co-occurrence of the gene and the toxin. These studies show that although the genes are necessary for toxin production, toxin is not always produced, and other factors are at play in the production of the toxins (e.g., environmental factors). These complicating factors are further indication that multiple linear regression modeling is a method to elucidate toxin triggers. One of the primary differences between this research and previous studies, was that lagged *anaC* and *sxtA* gene counts were used, and if genes precede toxin release by several days, it may account for the correlation and inclusion of the gene counts in the VB models.

VB has an option to calculate the wind direction in reference to beach orientation; however, three sites with different orientations were combined, so this additional calculation was not made. Nevertheless, wind direction was related to weighted-toxin concentration in the comprehensive MIX model, perhaps due to the direction of the prevailing wind being primarily toward a shoreline. In Kabetogama Lake, all three sampling locations are near a northern shoreline and a prevailing wind toward these shorelines could be described as northeasterly. Wind blowing toward shorelines has been shown to form surface scums and increase microcystin concentration (Wood et al., 2011) and wind mixes the water column and can alter light penetration, also leading to changes in the dominant cyanobacteria and thus toxins present. The best model included instantaneous wind direction rather than summing wind direction for any antecedent time period as described by other researchers (e.g., Francy et al., 2015).

Three variables were identified in the environmental MIX model including SC, LL_7day, and LL_Spring. These are the same variables identified in the environmental MC model and model parameter statistics were only slightly different. SC may be related due to the salinity effect on cyanobacterial cells, however, research is lacking on the effect of salinity on anatoxin-a

or saxitoxin (Christensen and Khan, 2020). Although blooms are more frequent at lower salinities (Li et al., 2015), some cyanobacteria can withstand higher salinity (Moustaka-Gouni et al., 2017).

This modeling effort indicated that a cyanotoxin mixture model and a microcystin-only model were not substantially different in terms of independent variables with similar evaluation statistics. However, the cyanotoxin mixture models were able to capture instances of anatoxin-a and saxitoxin occurrence. The microcystin model, only predicts microcystin occurrence. There were several instances where microcystin concentrations were below detection levels, yet either anatoxin-a or saxitoxin was present (Christensen et al., 2021a). The microcystin model cannot predict these instances of neurotoxin occurrence.

This effort also served as an indication of potential triggers for toxin production. However, there were limitations to this modeling effort, including the lack of consistent water-quality criteria or standards for the three toxins used in the model. Without national standards for individual or combined toxin concentrations in recreational water, there are multiple options for selecting a threshold value in VB. Beyond the model itself, there were limitations due to the data used: there were many censored values (those below the reporting limit) for several parameters, such as orthophosphorus and nitrite and, therefore, these and other parameters with greater than 85% censored values were eliminated as possible independent variables. Environmental variables such as PAR were measured at some distance from the sites, which is a limitation because weather conditions may be quite variable across even short distances. Moreover, the hydrology of this system is complicated and many inflows are unmeasured, which may lead to some uncertainty, including the uncertainty of flow direction (Christensen et al., 2016), which could have a significant effect on the relation between toxins and lake level variables during

years of unusually high or low flow. Finally, cyanotoxins may vary considerably within a bloom and throughout the day. Therefore, multiple samples collected throughout a bloom and at different times of the day, could have reduced the uncertainty.

4.4. Summary

Visitor use in Voyageurs National Park includes boating, fishing, and swimming, where such activities during cyanobacterial blooms could result in skin contact, ingestion, or inhalation of tiny water droplets containing the toxins. Children and dogs accompanying visitors may be at greater risk because of increased exposure and sensitivity. The park's lakes also support fish, loons, eagles, beaver, timber wolves, and moose that could be affected by the toxins. Owing to multiple concerns over human and ecosystem health, these models were developed as a preliminary step to understanding toxin triggers in Kabetogama Lake.

Blooms may often contain multiple toxins, but there is no known literature on models that predict a cyanotoxin mixture. This study presents four predictive models, two comprehensive and two using readily-available environmental variables only, for Kabetogama Lake in Voyageurs National Park. The models were developed with two years of data (2016-2017) collected from three recurring bloom locations. The comprehensive microcystin (MC) model had 6 variables with an R^2 of 0.87, whereas the comprehensive cyanotoxin-mixture (MIX) model had a best fit linear VB model with 6 variables and an R^2 of 0.86. Although the models were not significantly different in terms of R^2, the comprehensive MC model produced one false negative compared to zero false negatives for the MIX model. A model with false negatives may lead to a potentially serious scenario (Cyterski et al., 2015) with observations above the regulatory guidelines that were not simulated or predicted by the model.

The environmental variables for the MC and MIX models were similar with the same three independent variables in the best fit linear VB models. The best fit environmental MC model resulted in and R^2 of 0.58, whereas the best fit environmental MIX model resulted in an R^2 of 0.57. The quality of the environmental MC and MIX models are not significantly different, given the current data available for the Voyageurs National Park area; however, during years of more anatoxin-a or saxitoxin occurrence this relation could be different and a MIX model may be more accurate. Additionally, although these environmental models do not explain as much variability as the comprehensive models, they require less data and may serve as early-warning indicators because they include only variables for which data are readily available. Early warning of potential cyanobacterial toxins may give resource managers the ability to inform the public of the threat before visitors engage in water-based activities. This is the first known study on the use of Virtual Beach software for a cyanotoxin mixture model and although these models were developed for Kabetogama Lake in Voyageurs National Park, the methods used in this study may be applicable to other lakes or beaches.

CHAPTER 5: CYANOBACTERIAL SECONDARY METABOLITES AND THEIR RELATION TO PHYTOPLANKTON COMMUNITIES IN RECURRING SURFACE BLOOMS

5.1. Introduction

Cyanobacteria can produce secondary metabolites, including cyanotoxins, that are a concern in recreational waters. Numerous cyanotoxins exist in lakes used for recreation, including hepatotoxins, dermatoxins, and neurotoxins. Moreover, cyanobacteria capable of producing different toxins can be present at the same time (Christensen et al., 2021b; Christensen and Olds, 2019). However, most research to date has focused on the hepatotoxin microcystin, most often microcystin-LR. Less emphasis has been placed on various lesser studied toxins and secondary metabolites. Therefore, three recurring bloom sites in a remote temperate lake were studied to characterize the phytoplankton and cyanobacterial communities and a suite of lesser studied secondary metabolites, including cyanotoxins.

Voyageurs National Park, along the Minnesota (USA) and Canada border, was officially recognized as the 36th national park in 1975 (Zenzen, 2017), and in 1977 recurring blooms of cyanobacteria were documented in the park's interconnected lakes (Payne, 1991). In 2008, the cyanotoxin microcystin was identified in shallow and mesotrophic to eutrophic Kabetogama Lake (trophic state index = 44-54; Christensen et al., 2011), on the park's southern border (Christensen et al., 2011). Further research on Kabetogama Lake has advanced the understanding of recurring blooms, including documentation of neurotoxins, the seasonal variation in the phytoplankton community and toxin occurrence, differentiation between toxin-producing strains and non-toxic strains (Christensen et al., 2019), occurrence of toxins in fish tissue (Mcwhorter, 2020), and the development of a cyanotoxin mixture model (Christensen et al., 2021b). Although

the cyanobacteria that occur in these lakes are capable of producing numerous secondary metabolites, secondary metabolite data for Kabetogama Lake are limited to microcystin-LR, anatoxin-a, saxitoxin, and cylindrospermopsin (Christensen et al., 2011, 2019). Moreover, the toxicity of lesser-known metabolites, such as anabaenopeptins, are not well understood. This chapter covers research on phytoplankton and cyanobacterial communities, along with 19 toxins, including microcystin (11 congeners), nodularins, microginins, cyanopeptides (3 congeners), and anabaenopeptins (3 congeners) at three sites with recurring cyanobacterial blooms in Kabetogama Lake. Each toxins class is described below.

Anabaenopeptins are cyanopeptides with numerous variants (Cerasino et al., 2017), that often occur with microcystins (Lenz et al., 2019). Although anabaenopeptins were previously considered non-toxic (Beversdorf et al., 2017), sublethal effects have been reported. These sublethal effects include the inhibition of protease (an ezyme that helps digestion; Janssen, 2019) and adverse effects on the reproduction of nematodes from certain anabaenopeptin variants (Lenz et al., 2019). Anabaenopeptins are produced by multiple genera of cyanobacteria (Beversdorf et al., 2018), including *Microcystis aeruginosa* (Saker et al., 2005). Anabaenopeptins occur more frequently than microcystins (Beversdorf et al., 2017), but have not received much research attention in terms of potential human or ecological effects (Janssen, 2019).

Cyanopeptolins are cyanopeptides with numerous metabolites, including those produced by strains of *Microcystis aeruginosa*, *Planktothrix rubescens*, and *Tychonema bourrellyi* (Cerasino et al., 2017). Previously considered non-toxic, cyanopeptolins have been shown to induce DNA damage (Faltermann et al., 2014) and produce neurodevelopmental effects in zebrafish models (Zhang and Zhao, 2018). This toxicity may be a concern because

cyanopeptolins can occur as frequently as microcystins in surface waters (Janssen, 2019) and concentrations entering drinking water treatment plants in the U.S. Great Lakes Basin were similar to microcystin concentrations (Beversdorf et al., 2018).

Microcystins are a class of toxins that includes up to 250 congeners (Spoof et al., 2020), the toxicity of which has been determined on a small subset, for which there was a wide spectrum of potency and toxic effects in mice (Chernoff et al., 2020). Microcystins, one of the most abundant groups of cyanotoxins in the world (Roué et al., 2018), are found primarily in freshwater and produced by strains of cyanobacteria including *Microcystis*, *Dolichospermum*, and *Planktothrix* (Chorus and Welker, 2021; Merel et al., 2013).

Less is known about nodularins, another cyanobacterial hepatotoxin (Loftin et al., 2007) that is structurally similar to microcystin (Roué et al., 2018). Nine naturally occurring variants have been identified (Codd et al., 2005; Merel et al., 2013; Van Apeldoorn et al., 2007). Nodularins are found in brackish waters and produced by the cyanobacteria *Nodularia spumigena* (Van Apeldoorn et al., 2007).

Microginins are a class of cyanopeptide that can be produced by *Microcystis aeruginosa* (Stewart et al., 2018), *Nostoc oryzae*, *Synechococcus* sp. (Zervou et al., 2020), and *Nostoc* (Riba et al., 2020). A multitude of variants exist (Janssen, 2019; Stewart et al., 2018; Zervou et al., 2020), the toxicity for which range from non-toxic (Beversdorf et al., 2017), to slight toxicity for lower level organisms, having an effect on the reproduction and life span of worms (Lenz et al., 2019).

Previous studies have noted relations between cyanotoxins and environmental factors such as nutrients (Heisler et al., 2008) and low salinity (Li et al., 2015) and research at Voyageurs National Park has correlated cyanotoxin concentrations with wind direction, water

levels, specific conductance, water temperature, and other variables (Christensen et al., 2021). Previous studies also have noted a relation between microcystin concentrations and total phytoplankton biomass (Beaver et al., 2018), total phytoplankton abundance (Beaver et al., 2014), as well as the occurrence of individual taxa (Beaver et al., 2018; Kotak et al., 1995; Lee et al., 2015b). However, literature is sparse on any relations between these phytoplankton measures and microcystin congeners (other than microcystin-LR) or other lesser-known toxins.

The study described in this chapter is a first step toward filling the knowledge gap on lesser-known toxins, in addition to expanding the research on recurring blooms in Voyageurs National Park. It addresses the co-occurrence and concentrations of a suite of low-polarity cyanotoxins at three bloom sites in Voyageurs National Park. Phytoplankton occurrence, abundance, and biovolume were measured simultaneously in August and October 2019. The aims were to (1) determine the co-occurrence between microcystins and other low-polarity toxins, (2) compare toxin concentrations and phytoplankton communities between sites, and (3) identify phytoplankton (cyanobacteria) taxa indicative of the occurrence of microcystin and other toxins. Additional gaps exist in understanding of the mechanisms that regulate changes in bloom community composition; therefore, this study provides unique insight into how various cyanobacteria are related to each other and how combinations of cyanobacteria are related to toxins and toxin mixtures in recurring blooms.

5.2. Methodology

5.2.1. Water Sample Collection and Processing

Water sampling was conducted on August 21 and October 17, 2019 at each site (Fig. 4.1), which are roughly between 10 and 20 kilometers apart and only accessible by boat. Grab samples were collected less than 0.5 m below the surface using dip sampling procedures

(Graham et al., 2008). Six samples for phytoplankton identification and abundance were collected with 250-mL wide mouth plastic bottles and treated with 9:1 Lugol's iodine:acetic acid solution and refrigerated until shipment to BSA Environmental Service, Inc. (Beachwood, Ohio). Six samples for cyanotoxin analyses were collected by filling 500-mL amber glass bottles halfway and storing in a freezer at <-4°C until shipment via commercial-carrier next-day delivery to the Laboratory for Aquatic Environmental Microbiology and Chemistry at the University of Wisconsin-Milwaukee.

5.2.2. Laboratory Analyses

Phytoplankton were enumerated to the lowest possible taxonomic level using the membrane-filter technique (McNabb, 1960). This technique preserves cell structure so that samples can be examined and photographed at high magnifications with good resolution needed for taxonomic identification. A minimum of 400 units were counted from each sample according to methods described in Lund et al. (1958), which provided accuracy within 90 % confidence limits. Taxa identified with "cf" (confer or compare) were typically assigned as such because some critical structure or trait could not be identified due to specimen condition, rather than for a new or unique taxon (John Beaver, BSA Environmental Services, written commun., March 2, 2021). Additional detailed information on phytoplankton analyses are available from the U.S. Environmental Protection Agency (2012).

For cyanotoxin water samples, low-polarity toxins (Table 5.1) were analyzed using methods described by Bartlett et al. (2018). Briefly, after thawing, 2 mL of 100% methanol was added to reach a 67% MeOH extraction concentration and the extract sonicated at 45°C for 10 minutes (SharperTEK Stamina XP Heated Ultrasonic Cleaner, Pontiac, Michigan), then

centrifuged at 5000Xg for 15 minutes. Supernatants were stored in amber vials at -20°C until analysis. More information on the extraction method is available from Beversdorf et al. (2017).

Table 5.1. Secondary metabolites (toxins) analyzed in water samples by liquid chromatography tandem mass spectrometry (LC/MS-MS), their abbreviations, and known producers.

Secondary Metabolite	Abbreviation	Examples of Known Producers
Anabaenopeptin-F	AptF	*Microcystis aeruginosa* (Saker et al., 2005)
Anabaenopeptin-A	AptA	
Anabaenopeptin-B	AptB	
Cyanopeptolin-1020	C1020	*Microcystis aeruginosa*, *Planktothrix rubescens*, and *Tychonema bourrellyi* (Cerasino et al., 2017)[1]
Cyanopeptolin-1041	C1041	
Cyanopeptolin-1007	C1007	
Microcystin-RR	MCRR	*Anabaenopsis arnoldi* (MCRR, MCYR; Mohamed and Al Shehri, 2009), *Aphanocapsa cumulus* (Domingos et al., 1999)[1], *Aphanizomenon flos-aquae* (MCLR; Maatouk et al., 2002), *Arthrospira fusiformis* (MCYR; Ballot et al., 2005), *Chroococcus* (Jungblut and Neilan, 2006)[1], *Dolichospermum* (*Anabaena*; Li et al., 2016)[1], *Microcystis aeruginosa* (MCLR, Vezie et al., 1998; MCLF; Azevedo et al., 1994; MCLY, LeBlanc et al., 2020; MCLW, Robillot et al., 2000), *Microcystis* sp. (MCLA; Zastepa et al., 2015; MCWR; Namikoshi et al., 1992), *Microcystis viridis* (MCRR; Song et al., 2006), *Oscillatoria agardhii* (MCHtyR, Sano et al., 1998), *Phormidium* sp. (dmLR; Wood et al., 2010), *Planktothrix rubescens* (MCHi1R, Sano et al., 2004), *Pseudanabaena* (Kling et al., 2012)[1]
Microcystin-YR	MCYR	
Microcystin-LR	MCLR	
Microcystin-LA	MCLA	
[Dha7] microcystin-LR	dmLR	
Microcystin-LF	MCLF	
Microcystin-LY	MCLY	
Microcystin-WR	MCWR	
Microcystin-HilR	MCHilR	
Microcystin-HtyR	MCHtyR	
Microcystin-LW	MCLW	
Nodularin	NODN	*Nodularia spumigena* (Van Apeldoorn et al., 2007)
Microginin-690	Mg690	*Microcystis aeruginosa* (Stewart et al., 2018), *Nostoc oryzae*, *Synechococcus* sp. (Zervou et al., 2020), and *Nostoc* sp. (Riba et al., 2020)

[1]Taxa produced the toxin, but specific congeners were not identified.

The cyanotoxins were measured in 20 microliter (μL) injections using liquid chromatography-tandem mass spectrometry (LC−MS/ MS) with electrospray ionization. Analytes were separated on a C18 column using a Shimadzu Prominence HPLC and quantified on a Sciex 4000 QTRAP with electrospray ionization using a scheduled multiple-reaction monitoring method, and the following mobile phases: HPLC water with 0.1% formic acid and 5 mM ammonium acetate; and 95% acetonitrile with 0.1% formic acid and 5 mM ammonium acetate. Mass spectrometry conditions were optimized separately for each toxin and details are

available from Beversdorf et al. (2017). Minimum reporting limits were 0.01 microgram per liter (μg/L) for all toxins. Laboratory replicate and spiked matrix samples were analyzed with each batch and considered acceptable at a relative standard deviation (% RSD) equivalent to +/- 20% of average or expected value or less.

5.2.3. Statistical Analyses

For biological data, taxa identified with "cf" (confer or compare) were assumed to be that taxa and the cf was removed before statistical analysis. The Jaccard (1912) and Sorensen (1948) indices were used to ascertain whether phytoplankton community structure in each bloom was influenced by differences in site locations or sampling date. For these two indices, taxa of the same genus were combined to reduce noise. Although numerous quantitative modifications of each index exist in the literature, the authors' original equations were used as follows:

$$\text{Jaccard Index} = a/(b+c+a) \qquad \text{(Equation 1)}$$

$$\text{Sorensen Index} = 2a/(B+C) \qquad \text{(Equation 2)}$$

where a is the number of taxa common to both samples or locations; b is the number of unique taxa in the first sample or location; c is the number of unique taxa in the second sample or location; B is the total number of taxa in the first sample or location, and C is the total number of taxa in the second sample or location. If all species are common, the indices would equal 1; if the two groups have no species in common, the score would be 0.

Additionally, the Bray-Curtis Index (Bray and Curtis, 1957) was used to account for biovolume data, that is, the biovolume for each taxa and not simply presence or absence of taxa. The equation is typically expressed in terms of dissimilarity as follows:

$$\text{Bray-Curtis Dissimilarity Index} = 1 - ((2A_{bc})/(S_b+S_c)) \qquad \text{(Equation 3)}$$

where A_{bc} is the sum of only the lesser biovolume for each taxon found in both samples or locations; S_b is the total biovolume of specimen in the first sample; and S_c is the total biovolume

of specimen in the second sample. However, in this paper the Bray-Curtis Index was expressed as similarity (Clarke and Warwick, 1994) and multiplied by 100 to obtain percentage:

$$\text{Bray-Curtis Similarity Index} = 100 \times ((2A_{bc})/(S_b+S_c)) \quad \text{(Equation 4)}$$

Biovolume data were used rather than abundance data because biovolume is generally considered a better reflection of ecological importance than abundance (Clarke and Warwick, 1994). Moreover, cyanotoxin researchers provided evidence that biomass, rather than abundance, is relevant in toxin risk assessment (e.g., Ibelings et al., 2014). The biovolume data was square-root transformed in PRIMER-e software (Version 7, PRIMER-e Ltd, Devon, United Kingdom), which reduces the importance of abundant or large taxa, so that less common taxa exert some influence on the similarity calculations (Clarke and Warwick, 1994). PRIMER-e also was used to perform non-metric multidimensional scaling (nMDS) for visualizing the relations between taxa. The Bray-Curtis Index was used to group the taxa into clusters of 40, 60, and 80% similarity. Finally, PRIMER-e software was used to calculate Spearman's rank correlations between taxa biovolume (transformed) and toxin concentrations.

5.3. Results and Discussion

5.3.1. Toxin Occurrence

Overall, 6 toxins or secondary metabolites (anabaenopeptin A, anabaenopeptin B, anabaenopeptin F, [Dha7] microcystin-LR, microcystin-LA, and microcystin-YR), with 13 total occurrences were detected in August samples and 6 toxins or secondary metabolites (anabaenopeptin A, aabaenopeptin B, [Dha7] microcystin-LR, microcystin-LA, microcystin-LR, and microcystin-YR), with 14 total occurrences, were detected in October samples (Table 5.2). Cyanopeptides, microginins, nodularins, and several microcystin congeners (microcystin-HilR, microcystin-HtyR, microcystin-LF, microcystin-LW, microcystin-LY, microcystin-RR, and

microcystin-WR) were not detected above minimum reporting levels for any sample and are not included in the following tables, figures, or analyses.

Table 5.2. Cyanotoxin detections in samples collected from three recurring bloom sites in Kabetogama Lake, Voyageurs National Park, 2019. All concentrations in micrograms per liter.

Sample ID	Date	Time	AptA	AptB	AptF	dmLR	MCLA	MCLR	MCYR
A1 (August)	8/21/19	16:20	6.11	14.9	0.149	0.166	0.052	<0.01	0.231
A4 (August)	8/21/19	18:30	0.467	2.27	<0.01	0.035	0.0372	<0.01	0.0558
A5 (August)	8/21/19	17:00	0.238	0.595	<0.01	<0.01	<0.01	<0.01	<0.01
A1 (October)	10/17/19	12:20	0.533	0.359	<0.01	<0.01	<0.01	<0.01	<0.01
A4 (October)	10/17/19	10:55	2.58	3.33	<0.01	0.0378	.0521	0.144	0.0428
A5 (October)	10/17/19	11:55	77.3	82.1	<0.01	2.13	0.234	0.314	2.91
Mean			14.5	17.3	<0.01	--	--	--	--
Median			1.56	2.80	<0.01	0.0364	0.0446	<0.01	0.0493

Microcystin-YR had the highest median concentration of the microcystin congeners analyzed in Kabetogama Lake (0.0493 μg/L, Table 5.2), and was more abundant than microcystin-LR. However, microcystin-LR and -RR congeners are the most commonly detected worldwide, as well as in the midwestern USA (Graham et al., 2010). It is not known if the greater abundance of microcystin-YR over microcystin-LR is a regional, local, or seasonal occurrence, or if this phenomenon would be sustained over a longer period.

Microcystin-LR was the only toxin analyzed that currently has regulatory guidelines. One sample (site A5, in October) exceeded the 0.3 μg/L U.S. Environmental Protection Agency (EPA) Drinking Water Health Advisory (U.S. Environmental Protection Agency, 2015) and no sample exceeded the 1 μg/L World Health Organization (WHO) Guideline for Drinking Water Quality (World Health Organization, 2003) for microcystin-LR. However, total microcystins (combined total of microcystin congeners) at A1 (August) exceeded both EPA and WHO guidelines, and the A4 sample in October exceeded the EPA guideline. No samples exceeded

EPA proposed recreational guidelines (4 μg/L; D'Anglada and Strong, 2016) or WHO recreational guidelines of 20 μg/L (Chorus and Bartram, 1999) during 2019.

Site A1, in Sullivan Bay, had higher microcystin and anabaenopeptin concentrations in August (Table 5.2), whereas A5 had higher microcystin and anabaenopeptin concentrations in October. Site A4 also had higher metabolite concentrations in October, with the exception of microcystin-YR, which exceeded the concentrations of all other microcystin metabolites for all samples during 2019. Concentrations of microcystin-YR, microcystin-LA, and [Dha7]microcystin-LR at site A5 (October) exceeded all other samples by approximately 10-fold.

5.3.2. Phytoplankton Community Structure and Abundance

The phytoplankton community in Kabetogama Lake included 211 phytoplankton taxa in August, of which 28 were cyanobacteria, and 109 phytoplankton taxa in October, of which 14 were cyanobacteria (Appendix A, Table A1). Site A1 contained the most taxa in August (105) and October (81), followed by site A4 (80 in August and 45 in October) and site A5 (62 in August and 26 in October). Bacillariophyta (diatoms) comprised the most taxa at all sites, followed by Chlorophyta at sites A1 and A4. At site A5, however, the number of cyanobacteria taxa exceeded Chlorophyta taxa (Table A1). The remaining divisions (Charophyta, Chrysophyta, Cryptophyta, Euglenophyta, Miozoa, Ochrophyta, and Xanthophyta) were rare, with no more than 7 taxa in any one sample.

The composition of a phytoplankton community is strongly influenced by variability in water quality (Beaver et al., 2018). However, there were differences in the phytoplankton communities at the three Kabetogama Lake sites (Fig. 4.1), despite the sites having similar water-quality characteristics as confirmed by nMDS analysis (Christensen et al., 2021b). Site A1

contained the most phytoplankton taxa in both August (105 taxa) and October (81 taxa; Appendix A1); and cyanobacteria at site A1 comprised the lowest (43% and 7%) biovolume during August and October, respectively, and the fewest cyanobacterial taxa overall when compared with the other sites. This would seem to support that diverse communities are believed to be more stable and resilient. In October, the greater phytoplankton biodiversity at site A1 also may have led to fewer metabolites detected in October (2 of 19) compared to sites A4 and A5 (both sites having 6 of 19). Cyanobacterial blooms in October are not unusual for Kabetogama Lake (LeDuc et al., 2020), although many lakes bloom earlier in the season in this region (e.g., Loftin et al., 2016b).

In terms of biovolume, cyanobacteria and Bacillariophyta (diatoms) dominated most samples. Cyanobacteria at the A1, A4, and A5 sites comprised 43%, 59%, and 50%, respectively, of the blooms in August, and 7%, 27%, and 95%, respectively, of the blooms in October (Fig. 5.1), indicating that cyanobacteria biovolume is similar among the August samples, but varies greatly in October. Site A5 is noticeably different from the other two sites, with 34% Miozoa in August, and 95% cyanobacteria in October.

Cell density in each division represents the number of algal or cyanobacterial cells per unit volume, calling attention to taxa with smaller cell volume that are large in number, such as in some cyanobacterial blooms in Kabetogama Lake (Christensen et al., 2011). In terms of cell density, cyanobacteria comprised the highest percentage (88-98%) of total sample density at all sites except the October A1 sample (32%; Appendix Table A1). Chlorophyta comprised the next highest density (2-8%) at all sites except the October A1 sample, where Cryptophyta comprised 33% of the sample, followed by cyanobacteria. Site A1 appeared to have recovered from the

August bloom and had a significant change in cyanobacterial density between the August and October samples.

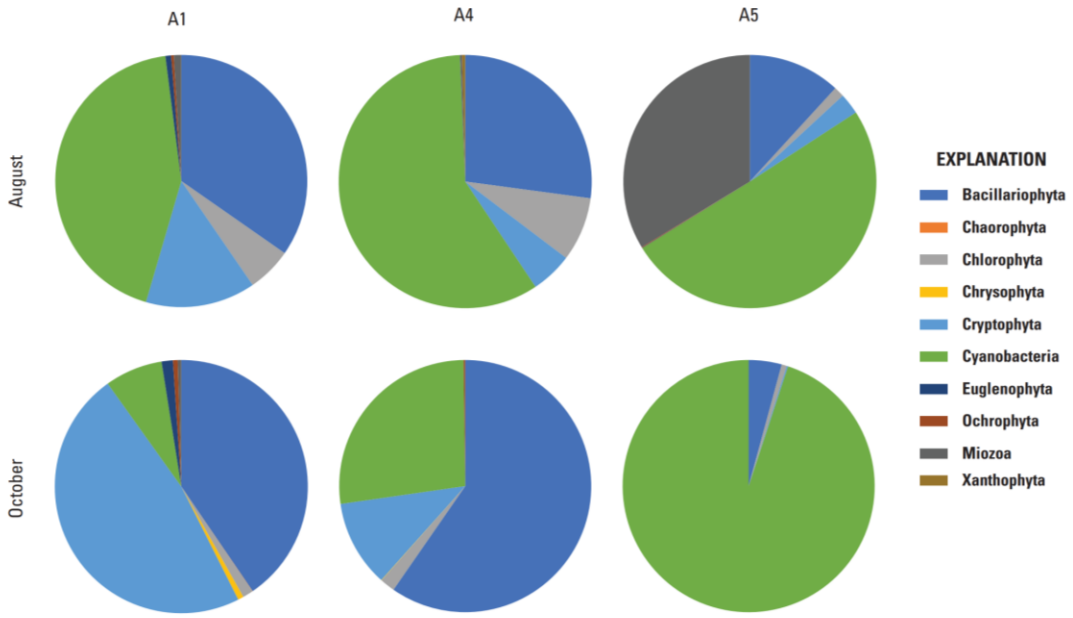

Figure 5.1. Biovolume in six phytoplankton samples collected from three sites in Kabetogama Lake, in August and October 2019.

The WHO established guidelines on total cyanobacterial abundance to assess recreational risk (Chorus and Bartram, 1999), considering cyanobacteria abundance to be low (<20,000 cells/mL), moderate (<100,000 cells/mL), high (<10,000,000 cells/mL), or very high (>10,000,000 cells/mL). These categories place the site A1 October sample into the high category and all other samples in the very high category. However, in a study of 1,161 lakes in the United States, the WHO cyanobacterial abundance guidelines overestimated microcystin risk (Loftin et al., 2016). Therefore, a more in-depth look at the cyanobacteria, including the toxin producing species, is warranted.

5.3.3. Patterns of Taxon and Toxin Similarity Between Sites and Samples

The number of unique phytoplankton was greatest at site A1 in Sullivan Bay, whereas the number of unique cyanobacteria was lowest at this site, with fewer species dominating the cyanobacterial community. The August A1 sample included the potential anabaenopeptin- and microcystin-producing taxa *Microcystis*, and the potential microcystin-producing taxa *Anabaenopsis* and *Pseudanabaena*. The microcystin-producing species of *Aphanizomenon*, *Chroococcus*, and *Dolichospermum* occurred at all sites and in all samples.

Jaccard's similarity indices among sites range from 0.36 to 0.89 (Table 5.3), indicating more similarity between sites A4 and A5, than between A1 and A4 or A5 (index values below about 0.50 indicate low similarity; values above 0.50 indicate more similarity). Slightly lower values for Jaccard's similarity index occurred between seasons (0.38-0.57). The Sorensen index, however, was less conclusive overall, with indices of 0.55-0.73 for seasonal similarity and 0.42-0.80 for similarity between sites; however, August samples were highly similar among sites (0.69-0.80). For all sites, Jaccard's similarity index and Sorensen's similarity index confirmed lower similarity between August and October blooms from the same site than between bloom samples collected on the same day from different sites.

Most of the phytoplankton (7 genera) and cyanobacteria (9 taxa) occurred at all three sites and an nMDS analysis on all phytoplankton showed few patterns (Fig 5.2A). However, similar to the Jaccard and Sorensen similarity indices, the nMDS on cyanobacteria alone indicated stronger similarity between seasons than between sites (Fig 5.2B), with the Site A1 sample from October strongly dissimilar from all other samples.

Table 5.3. Similarity indices for algal bloom samples from Kabetogama Lake, August and October 2019.

Index	Jaccard	Sorensen
Similarity Between August and October Samples		
A1	0.38	0.55
A4	0.54	0.70
A5	0.57	0.73
Similarity Between Sites, August Samples		
A1-A4	0.53	0.69
A1-A5	0.67	0.80
A4-A5	0.67	0.80
Similarity Between Sites, October Samples		
A1-A4	0.50	0.62
A1-A5	0.36	0.47
A4-A5	0.55	0.71
Similarity Between Sites, Combined August and October Samples		
A1-A4	0.55	0.42
A1-A5	0.69	0.55
A4-A5	0.89	0.57

Phytoplankton, cyanobacteria, and toxins that were unique to a sample were examined to gain a better understanding of their inter-relationships (Fig. 5.3). For August samples, site A1 had two unique cyanobacterial taxa (*Oscillatoria* and *Raphidiopsis*) and one unique metabolite (Anabaenopeptin F). Site A4 had one unique phytoplankton division (Xanthophyta), two unique cyanobacterial taxa (*Synechococcus* and *Woronichinia*), and no unique toxins. Site A5 had no unique divisions, taxa, or toxins. One cyanobacterium (*Microcystis*) was unique only to site A1 and A4, where 3 toxins also occurred (microcystin-YR, microcystin-LA, and [Dha7] microcystin-LR).

Figure 5.2. Non-metric multidimensional (nMDS) ordination showing (A) similarity between sites based on all phytoplankton taxa, and (B) similarity between sites based on cyanobacteria taxa only using Bray-Curtis Similarity Indices of square-root transformed biovolume data, Kabetogama Lake, 2019.

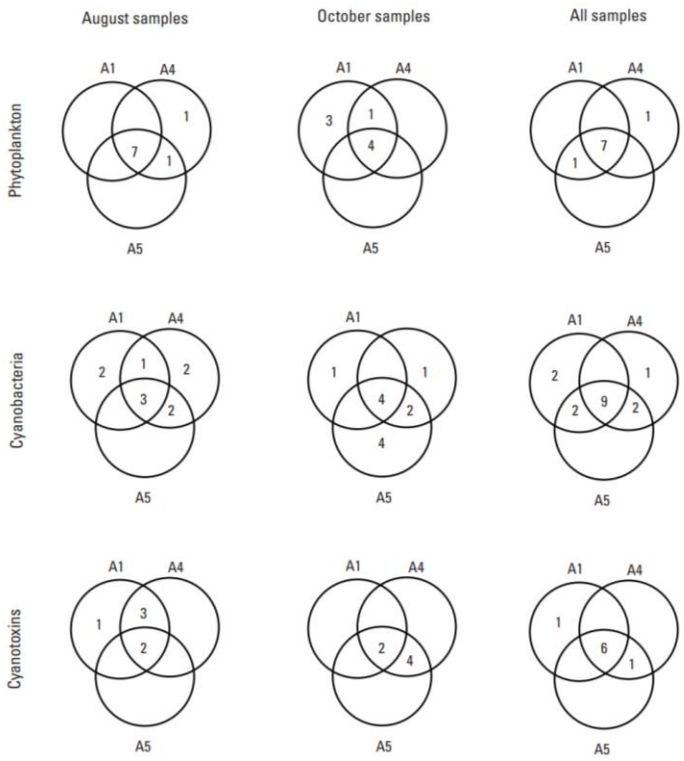

Figure 5.3. Venn diagrams showing number of common phytoplankton taxa, cyanobacterial taxa, and secondary metabolites among three sites in Kabetogama Lake, 2019.

For October samples, site A1 had three unique divisions (Euglenophyta, Ochrophyta, and Miozoa), one unique cyanobacteria (*Cylindrospermopsis*), and no unique toxins; site A4 had no unique phytoplankton, one unique cyanobacteria (*Synechococcus*) and no unique toxins; and site A5 had no unique phytoplankton, 4 unique cyanobacteria (*Anabaenopsis*, *Aphanocapsa*, *Microcystis*, and *Woronichinia*), and no unique toxins. Two cyanobacteria occurred at both site A4 and A5 (*Phormidium* and *Pseudanabaena*) that did not occur at site A1, along with 4 toxins ([Dha7] microcystin-LR, microcystin-LA, microcystin-LR, and microcystin-YR) that did not occur at site A1.

Site A1 is on the south side of the lake in the mostly enclosed Sullivan Bay (Fig. 4.1), whereas sites A4 and A5 are about 10-20 kilometers away on the north side. Site A1 also is near

Ash River and several resorts, which may be a source of nutrients. Therefore, the difference in bloom timing might be attributed to different sources, toxin producers, and the path of nutrients through the system in late summer and fall. However, all sites contained cyanobacteria that are capable of fixing nitrogen from the atmosphere (*Aphanizomenon*, *Chroococcus*, *Dolichospermum*, *Oscillatoria*, *Phormidium*, and *Raphidiopsis* sp.; De Nobel et al., 1998; Stewart, 1973) and Kabetogama Lake has been shown to supply phosphorus from internal loading (bottom sediment; Christensen et al., 2013). Therefore, both nitrogen and phosphorus are likely available to cyanobacteria in Kabetogama Lake, regardless of proximity to external nutrient sources and questions remain as to why the site A1 October bloom was substantially different from blooms at sites A4 and A5.

An additional nMDS was performed on the cyanobacterial taxa to get a visual representation of the relations among species (Fig. 5.4). Three taxa pairs occurred with 80% similarity among samples: *Raphidiopsis* sp. and *Oscillatoria* sp.; *Dolichospermum affine* and *Cuspidothrix* sp.; and *Aphanocapsa* sp. and *Eucapsis microscopica*. Additional taxa occurred with 60% similarity, including two groups of three taxa: *Pseudanabaena minima*, *Aphanocapsa* sp., and *Eucapsis microscopica*; and *Aphanizomenon* spp., *Chroococcus* spp., and *Pseudanabaena* spp.

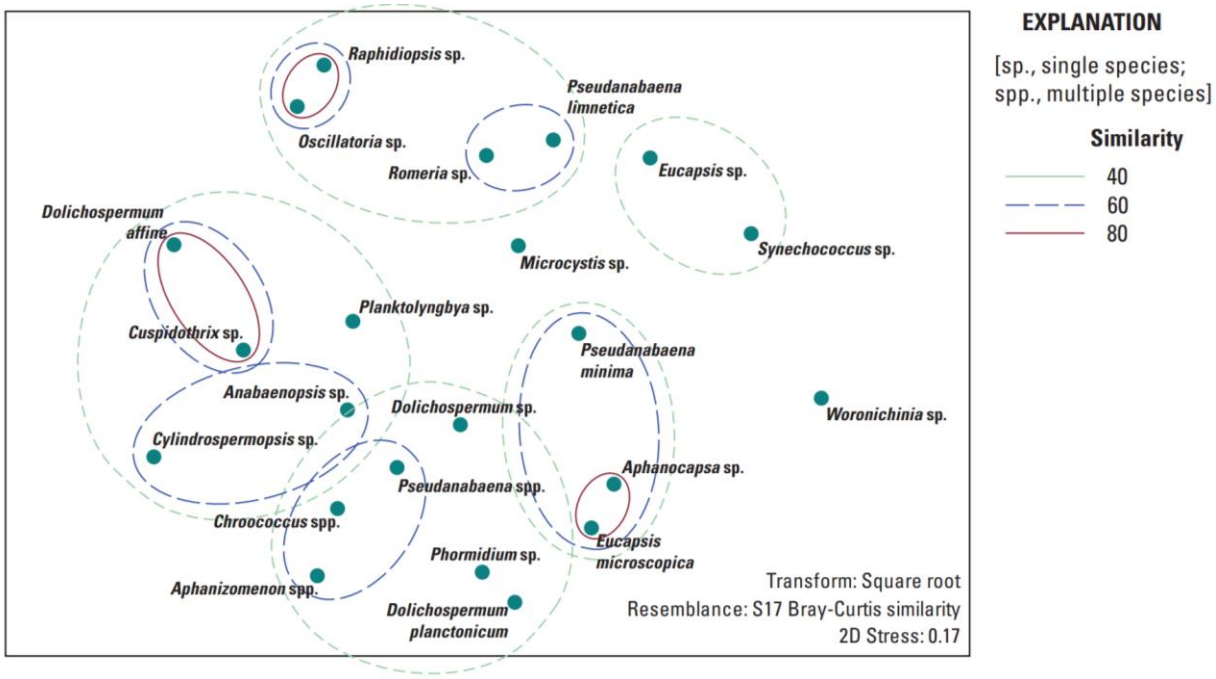

Figure 5.4. Non-metric multidimensional (nMDS) ordination showing similarity between taxa based on Bray-Curtis Similarity Indices of square-root transformed biovolume data, Kabetogama Lake, 2019.

The three taxa pairs occurring with 80% similarity in the nMDS ordination, may reflect either symbiotic or antagonistic relations. Some research indicates that nitrogen-fixers may form symbiotic relations with certain plants (e.g. Chernoff et al., 2017) in order to obtain the needed nitrogen for growth and survival; non-nitrogen fixing cyanobacteria may be associated with nitrogen-fixing cyanobacteria for similar reasons.

Raphidiopsis may fix nitrogen under microaerobic conditions (Stewart, 1973), but only has heterocytes during certain life stage (Moustaka-Gouni et al., 2009), whereas *Oscillatoria* does not have heterocytes and therefore does not fix nitrogen from the atmosphere (Paerl and Otten, 2016; Rosen and St. Amand, 2015). Another pair with 80% similarity, *Dolichospermum* and *Cuspidothrix*, both contain heterocytes (Rosen and St. Amand, 2015; Wacklin et al., 2009), and thus are both capable of fixing nitrogen from the atmosphere. *Aphanocapsa* sp. and *Eucapsis*

microscopica do not contain heterocytes and thus are incapable of fixing nitrogen. Although evidence of symbiotic relations is not strongly supported by the limited data from Kabetogama Lake, it is possible that the clustering habit of these species within a bloom aids in nutrient recycling, enabling more taxa to prosper under nitrogen-depleted conditions. Additional data collection may help clarify symbiotic or antagonistic links between taxa or identify taxa that co-dominate in certain conditions as functional groups (Becker et al., 2010).

To further explore the relations between cyanobacteria and cyanotoxins, Spearman's rank correlations were computed between every cyanobacterial taxa and toxin that occurred in more than one sample (Table 5.4). Surprisingly, potential microcystin-producing species *Aphanizomenon*, *Chroococcus*, and *Dolichospermum* were not statistically positively related to any of the microcystin congeners. However, the potential microcystin producer *Microcystis* was significantly correlated to microcystin-YR.

Table 5.4. Spearman's rank correlations between cyanobacterial taxa and secondary metabolites observed in Kabetogama Lake, 2019.

Cyanobacterial taxa	AptA	AptB	dmLR	MCLA	MCLR*	MCYR
Anabaenopsis sp.	0.58	0.67	0.63	0.69	0.55	0.37
Aphanizomenon sp.	0.60	0.26	0.31	0.20	0.34	0.26
Aphanocapsa sp.	-0.03	0.15	0.06	0.30	0.57	-0.18
Chroococcus sp.	0.66	0.14	0.29	0.23	0.27	0.23
Cuspidothrix sp.	0.44	0.44	0.45	0.38	0.02	0.27
Cylindrospermopsis sp.	0.21	0.33	0.34	0.40	0.07	0.22
Dolichospermum planctonicum	-0.21	0.03	-0.09	0.09	0.40	-0.28
Dolichospermum sp.	0.08	0.14	0.06	-0.06	0.10	0.00
Eucapsis sp.	-0.33	-0.10	-0.22	-0.39	-0.48	-0.22
Eucapsis microscopica	-0.30	-0.14	-0.22	0.02	0.22	-0.46
Microcystis sp.	0.58	0.70	0.65	0.46	0.35	**0.65**
Phormidium sp.	0.46	0.26	0.29	0.50	0.86	0.09
Planktolyngbya sp.	0.09	-0.03	0.06	-0.29	-0.37	-0.12
Pseudanabaena limnetica	0.21	0.39	0.31	0.06	-0.14	0.31
Pseudanabaena minima	-0.15	0.03	-0.06	0.06	0.07	-0.30
Pseudanabaena sp.	-0.14	-0.54	-0.49	-0.37	-0.10	**-0.72**
Pseudanabaena spp.	0.41	0.41	0.42	0.42	0.12	0.21
Romeria sp.	0.15	0.27	0.22	0.15	-0.11	0.03
Synechococcus sp.	**-0.68**	**-0.78**	**-0.82**	**-0.82**	-0.48	**-0.82**
Woronichinia sp.	0.17	0.34	0.25	0.26	0.50	0.26

Numbers indicate Spearman's rank correlation between taxa biovolume and metabolite concentration; white squares indicate no correlation (Spearman's rho less than +/-0.20; light blue squares indicate moderate positive correlation (rho 0.20-0.49); dark blue squares indicate high positive correlation $\geq$0.50; light red squares indicate moderate negative correlation (rho -0.20-0.49; dark red squares indicate high negative correlation (rho $\geq$0.50); refer to Table 5.1 for an explanation of abbreviations used; Numbers in bold indicate that the p-value was less than 0.1; *MCLR occurred in only two samples.

The negative correlations between some taxa and secondary metabolites are particularly interesting. *Pseudanabaena* sp. and *Synechococcus* sp. had strong negative correlations with several toxins. Hu et al. (2005) demonstrated that growth of *Synechococcus* was significantly inhibited by the presence of microcystin-RR, which is known to be produced by *Anabaenopsis* and *Microcystis* (Table 5.1). Although microcystin-RR was not detected in any sample, other microcystin congeners may have similar effects.

Phormidium and *Pseudanabaena* were unique to sites A4 and A5, along with 4 toxins ([Dha7] microcystin-LR, microcystin-LA, microcystin-LR, and microcystin-YR) that do not occur at site A1. Although these results do not confirm that the toxins are unique to a site or are produced by the unique cyanobacteria, it is a starting point for future research and may have an effect on citizen science and management actions. For example, *Anabaenopsis* may be a taxon to focus on, given that it is the only one that co-occurs with microcystin-LA.

Some researchers have developed metrics to assess the health of phytoplankton communities (e.g. Devlin et al., 2009; Longphuirt et al., 2019; Tett et al., 2008); however, these metrics were developed for other waterbodies and may not be applicable to a northern temperate lake. It is generally supported that large biovolumes or densities of cyanobacteria are cause for concern (Chorus and Bartram, 1999) and generally a "balance of organisms" is considered healthy (Tett et al., 2008). Thus, all bloom samples collected as part of this study may be a concern due to their high cyanobacteria counts and densities, although they represent the water quality at the bloom sites and not the entire lake.

There still is no agreement on why certain cyanobacteria produce secondary metabolites. Cyanotoxin production may be influenced by environmental and water-quality conditions, such as wind direction and lake level which were significant factors in predictive models for cyanotoxins in Kabetogama Lake (Christensen et al., 2021b), but cyanotoxin production may be influenced just as much by other phytoplankton and cyanobacteria, and these interactions may be strongly dependent on mixing events (Bertos-Fortis et al., 2016). Bray and Curtis (1957) reported that community is the key to biologic and physical phenomena; so mutual occurrence of certain taxa may stimulate metabolite production, whether the organism produces it as a defense or the two taxa work synergistically on survival. The associations presented here bring us one step

closer to understanding the complex relations among phytoplankton and cyanobacteria communities and the secondary metabolites they produce.

5.4. Summary

Some cyanobacteria produce secondary metabolites, with varying degrees of toxicity, and data on many secondary metabolites in recreational lakes are limited. Moreover, data were not available on cyanopeptides or microcystin congeners in Kabetogama Lake, other than microcystin-LR. However, microcystin congeners have displayed a range of toxicity in a variety of organisms (e.g., Chernoff et al., 2020) and their combined toxicity is unknown. Also unknown is the human toxicity of the cyanopeptides, although they may inhibit protease or protein phosphatases, thus altering human physiology (Beversdorf et al., 2018).

Therefore, one of the aims of this study was to determine the co-occurrence between microcystins and other low-polarity metabolites. For this aim, phytoplankton, cyanobacteria, and 19 low-polarity secondary metabolites at three sites with recurring blooms in Kabetogama Lake within Voyageurs National Park were investigated. Seven of 19 secondary metabolites were detected (anabaenopeptin A, anabaenopeptin B, anabaenopeptin F, [Dha7] microcystin-LR, microcystin-LA, microcystin-LR, and microcystin-YR) in various mixtures. Anabaenopeptin A and B were detected in every sample. Microcystin-YR was detected more frequently and at higher concentrations than microcystin-LR, unlike other lakes in the region, although concentrations generally were low with only two samples exceeding either U.S. Environmental Protection Agency or World Health Organization total microcystin drinking water guidelines and no samples exceeding recreational guidelines. This study examined only a small portion (19) of the hundreds of cyanobacterial metabolites known to exist.

A second aim of this study was to compare toxin concentrations to the phytoplankton communities between sites. Many phytoplankton and cyanobacteria occurred at all three sites. Yet there were differences in the phytoplankton communities at the three spatially distant Kabetogama Lake sites, with site A1 in Sullivan Bay having the greatest number of phytoplankton taxa, corresponding with a lower number cyanobacterial taxon. Jaccard's similarity index among sites ranged from 0.36 – 0.89, and indicated lower variability between sites A4 and A5, than between A1 and A4 or A5. Lower values for Jaccard's similarity index between seasons (0.38-0.57) indicated that variability was higher between blooms occurring in different seasons at the same site than between bloom samples collected on the same day from different sites.

The third aim of this study was to identify cyanobacteria indicative of the occurrence of toxins or other secondary metabolites. Potential microcystin-producing species *Aphanizomenon*, *Chroococcus*, and *Dolichospermum* were not statistically related to any of the microcystin congeners. *Microcystis* was significantly correlated to microcystin-YR and *Pseudanabaena* sp. and *Synechococcus* sp. were negatively correlated to several toxins.

Nitrogen-fixing cyanobacteria were present at every site, and when combined with internal loading of phosphorus known to occur in the lake, might explain the taxa and toxin similarities between sites, and why seasonal differences were stronger. This study sheds some light on the interrelations of various phytoplankton and cyanobacteria in the recurring blooms in Kabetogama Lake.

CHAPTER 6: OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

6.1. Overall Conclusions

Kabetogama Lake in Voyageurs National Park, Minnesota, USA suffers from recurring late summer algal blooms that often contain toxin-producing cyanobacteria. By sampling a recurring bloom location throughout the entire open water season in 2016 (Chapter 3), this research showed that anatoxin-a was detected in only one sample two weeks before the microcystin peak concentration, and that the summer peak concentration of saxitoxin occurred about a week before microcystin peak concentrations. Moreover, toxin-forming cyanobacteria were present before visible blooms, indicating that sampling for additional toxins and sampling earlier in the season may be necessary to assess ecosystems and human health risk.

This research presented two cyanotoxin mixture models (MIX) and compared them to two microcystin (MC) models from data collected in 2016-2017 from three recurring cyanobacterial bloom locations in Kabetogama Lake (Chapter 4). Models included those using near-real-time environmental variables (readily available) and those using additional comprehensive variables (based on laboratory analyses). Comprehensive models explained more variability than the environmental models and neither MIX model was a better fit than the MC models. However, the MIX models produced no false negatives in the calibration dataset, indicating that all observations above human-health regulatory guidelines were simulated by the MIX models.

In 2019, phytoplankton, cyanobacteria, and low-polarity secondary metabolites were examined at the three sites in Kabetogama Lake to help understand biological community interactions and toxin co-occurrence (Chapter 5). Seven of 19 toxins were detected in various mixtures: anabaenopeptin A and B were detected in every sample and microcystin-YR was

detected more frequently and at higher concentrations than microcystin-LR, unlike other lakes in the region, although concentrations generally were low with only two samples exceeding either U.S. Environmental Protection Agency or World Health Organization total microcystin drinking water guidelines and no samples exceeding recreational guidelines. The potential toxin producing cyanobacteria, *Microcystis*, was significantly correlated to [Dha7] microcystin-LR and microcystin-YR. Anabaenopeptins correlated with six taxa, most of which have no literature available on whether they are capable of producing these secondary metabolites. Jaccard and Sorenson indices indicated that phytoplankton and cyanobacterial communities were more similar among different site locations on the same date than samples from the same site but different seasons. Nitrogen-fixing cyanobacteria were present at every site, and when combined with internal loading of phosphorus, might explain the taxa and toxin similarities between sites, and why seasonal differences were stronger.

6.2. Future Research Directions on Neurotoxins and Other Cyanotoxins

Although this research adds to the body of work on under-studied cyanotoxins and secondary metabolites, particularly in recurring blooms, several additional research gaps remain. Research gaps and new directions presented below are given in two parts, first the research gaps for the field of cyanotoxins and neurotoxins in freshwater, followed by new directions for research at Voyageurs National Park.

6.2.1. Occurrence and Biogeography

Other than Australia and New Zealand, few studies on neurotoxins or under-studied secondary metabolites have taken place south of the equator. In addition, the expense of analytical methods may preclude less affluent nations from access to anatoxin-a and saxitoxin data collection and laboratory analysis. Many studies would benefit from targeted monitoring for

neurotoxins, in addition to microcystin, in freshwater environments that have the cyanobacteria capable of producing neurotoxins in combination with the optimal environmental conditions for their production and release. Moreover, in freshwater environments where neurotoxins are a known problem, sampling when cyanobacteria are not visibly present may be important. Fastner et al. (2018) reported no visible blooms for an event where anatoxin-a was implicated in dog deaths, and the lack of a bloom does not warrant forgoing sampling if other conditions are optimal. Most research is performed in response to a human health issue and thus in populated areas. Research in remote locations might increase our knowledge base on the biogeography and ecosystem effects of cyanotoxins in areas with little human influence.

6.2.2. Triggers of Toxin Production and Release

The collection and reporting of ancillary data, along with toxin analysis, may help pinpoint the triggers of toxin production and release. This dissertation covered the role of salinity, temperature, sunlight, pH, and nutrients on neurotoxins and the role of wind and water levels on microcystin and cyanotoxin mixtures, yet very few studies have reported on these conditions or related data such as specific conductance. The triggers of toxin production and release may lead to a better understanding of when and where exposure is most likely.

6.2.3. Environmental Fate and Degradation

Whereas anatoxin-a degrades rapidly in sunlight, no studies look at saxitoxin under sunlight, PAR, or UVB. Additionally, few studies addressed the fate of cyanotoxins in soil, either benthic or terrestrial.

6.2.4. Environmental Exposure Routes

Some environmentally relevant exposure routes have not been examined thoroughly in the literature. Many studies assume exposure through drinking water, inadvertently during

recreational activities, or consuming contaminated fish or shellfish. However, aspirating the cyanotoxins is a possible exposure route. For brevetoxin, a neurotoxin found in marine environments, Buttke et al. (2017) hypothesized that the toxin was aerosolized and transported inland via wind during a storm, in rainfall, or in insects, resulting in the confirmed mortality of green tree frogs from brevetoxin, and the likely mortality of ground squirrels and a coyote. Trainer and Hardy (2015) reported that saxitoxins are toxic by inhalation as well as ingestion. This exposure route, among others, is an important potential source for humans and other animals. Another substantial gap is the understanding of the effects of secondary exposure (e.g., the consumption of fish or shellfish by humans) in freshwater environments. To this end, food preparation methods (e.g., cooking) also may affect toxin survival and exposure. Understanding some of these secondary exposure routes will lead to an increased understanding of the effects higher in the trophic chain.

6.2.5. Seasonal and Diel Variation in Toxicity

Given potential changes in climate, changes in peak cyanotoxin production may be expected. Late fall toxicity could be a concern for migratory birds (Rose, 1953), but few data are available, as sampling outside of warm summer months is not common in temperate climates. Diel variability also is an important factor in exposure risk, considering that anatoxin-a is degraded in sunlight in as little as one hour. In contrast, microcystin concentrations are highest during daylight hours (Kotak et al., 1995). The apparent opposite diel patterns of anatoxin-a and microcystin highlight the importance of collecting samples throughout a 24-hour cycle. Whereas diel changes in oxygen levels (e.g. Rose, 1953) and in the physiology of cyanobacteria (Welkie et al., 2019) have been considered in the literature, no research on diel changes in toxicity was found.

6.2.6. Food Web Effects

Many studies have looked at food web effects of saxitoxin in marine environments, but similar studies in freshwater are rare. Those studies that do exist are primarily focused on microcystin (e.g., Vanderploeg et al., 2001), although freshwater toxin studies, particularly on freshwater mussels because of the similarity with marine mussels, would be beneficial. One key research gap concerns studies that involve either simple food chains or complete natural communities to examine sublethal effects of neurotoxins and other cyanobacterial toxins on populations or ecosystems.

6.2.7. Toxicological Studies of Cyanotoxin Mixtures

Multiple toxin-producing strains of cyanobacteria can co-exist (Ferreira et al., 2001), and multiple toxins can be produced by a bloom. Synergistic or antagonistic effects of neurotoxins and other secondary metabolites that co-occur are not well characterized. Laboratory exposure studies for multiple toxins may be beneficial. The research presented in this dissertation showed that peak microcystin concentrations did not coincide with peak saxitoxin concentrations, whereas other studies documented that peak microcystin concentrations did not coincide with peak anatoxin-a (Boyer 2008). Graham et al. (2010) reported multiple classes of cyanotoxins in about 48 percent of all bloom samples, which has important implications for the many studies that only test for microcystins.

6.2.8. Toxicological Studies of Sublethal Health Effects

Toxicological studies identifying long-term sublethal and chronic health effects are a gap in the current research on freshwater cyanotoxins, particularly the neurotoxins. From animal models, potential adverse health effects of anatoxin-a and saxitoxin are numerous, ranging from mild to severe. Although there have been no reported human deaths from freshwater neurotoxin

exposure, with the exception of the case in Wisconsin, USA (Behm, 2003), many studies agree on a lethal human dose of about 200-250 μg/kg for anatoxin-a and about 10 μg/kg for saxitoxin. However, most people are not exposed to this concentration when participating in recreational activities in the water, such as swimming and boating. Acute poisonings and death may occur; however, chronic exposure is more likely. Therefore, studying chronic and sublethal effects would more adequately address ecosystem effects and human safety.

6.2.9. Biological Influences on Toxin Production

This dissertation revealed some interesting relations among cyanobacteria and cyanotoxins, particularly the under-studied neurotoxins and low-polarity secondary metabolites. The relations between certain taxa and toxins found in the natural environment, where numerous factors may influence those interactions, are not definitive evidence that these taxa are producing toxins or that taxa are acting antagonistically or synergistically. However, this dissertation identifies taxa and toxins for prospective laboratory experiments where these mechanisms can be confirmed. Moreover, additional research on rare taxa may be beneficial. Despite their rare occurrence in blooms, rare taxa may play a key role in cyanobacterial succession, and eventual toxin production.

6.3. Future Research Directions in Voyageurs National Park

In addition to these research gaps, the research and modeling completed at Voyageurs National Park indicated a few gaps that are worthy of future work. Here are three research ideas that have received funding for work in 2021 and beyond.

6.3.1. Study on Diel Variability of Toxins

It would be beneficial to examine the diel variability of cyanotoxins, and anatoxin-a in particular. A 24-hour study of cyanotoxins in the park has been proposed and funded. Several

dog and cattle deaths in the region indicate that the potent neurotoxin, anatoxin-a, is a concern in pre-dawn hours and on cloudy days and the only suspected human anatoxin-a death occurred following an after-dark swim (Behm, 2003). However, most sampling is completed midday. Sampling neurotoxins and other cyanotoxins over 24-hour periods, along with photosynthetically active radiation, would be one avenue to assess the relation between sunlight and these potent toxins, so that resource managers can make more informed decisions about recreational water use.

6.3.2. Study on Water-level Manipulation

Because water levels can be manipulated at downstream dams, affecting park waterbodies, a future study will examine the potential for alternate water-level management plans (rule curves) to reduce cyanobacterial blooms and resulting toxicity in Lake Kabetogama. The rule curves for the dams that regulate the park's waterbodies are updated periodically to reflect new research and understanding of the system and to promote desirable ecological outcomes. Measuring the effect of drying and re-wetting of shoreline sediment on nitrogen and phosphorus released, may result in further understanding of how water levels impact cyanobacteria and toxin release.

6.3.3. National-level Study on How to Rapidly Assess Toxins in National Parks

There will be a collaboration with the National Park Service's Office of Public Health, the U.S. Environmental Protection Agency's Cyanobacteria Monitoring Collaborative, and National Oceanic and Atmospheric Administration's Phytoplankton Monitoring Network to supply equipment and protocols for low-cost toxin sampling methods needed for analysis of up to 32 freshwater and 25 marine toxins, which will provide the data essential to establish management action thresholds for algal blooms in National Parks. To date, much of the data

collection in and outside of the parks is performed for different purposes (e.g., assessing toxin triggers, determining sources, or assessing responses to climate change). These individual, locally focused harmful algal bloom and cyanotoxin studies have led to a patchwork of sample designs, analyses, and management strategies. As a result, the data generated are not readily summarized into a concise assessment of cyanobacterial blooms across parks. New techniques such as remote sensing to detect the presence of blooms show promise, but such techniques cannot detect toxins—the most important aspect of cyanobacterial blooms from a human and wildlife health perspective. With no coordinated process for when and how to sample blooms for toxins and the lack of knowledge of under-studied toxins, there is a need for protocols and additional methods that are cost effective and achievable by parks with few staff and/or engaged citizen science groups.

Despite the rapidly expanding literature on cyanotoxins, there is still a lack of understanding of why cyanobacteria produce toxins and if these toxins serve any ecological function. Work will continue at Voyageurs National Park and elsewhere to help answer these and other remaining questions. Further research will help scientists understand some of the lesser-known and rarely studied toxins produced by cyanobacteria, the ubiquitous organisms that have had major effects on the aquatic environment, the atmosphere, and on the Earth.

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APPENDIX. SUPPLEMENTARY TABLE

The following table consists of phytoplankton data from water samples collected at Kabetogama Lake in Voyageurs National Park and analyzed by BSA Environmental Services, Inc. (Beachwood, Ohio). Samples were collected at recurring bloom locations, at the water surface. Sampling, processing, and laboratory methods followed those described in Christensen et al. (2019).

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park, 2019.

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A1, August 21, 2019 16:15				
cf. *Achnanthes* sp.	Bacillariophyta	1	8.52E+04	1.75E+07
Achnanthidium cf. *exiguum*	Bacillariophyta	1	8.52E+04	1.16E+07
Asterionella formosa	Bacillariophyta	5	1.33E+05	3.94E+07
Aulacoseira alpigena	Bacillariophyta	6	5.11E+05	1.63E+08
Aulacoseira granulata	Bacillariophyta	21	1.79E+06	1.19E+09
Cocconeis cf. *placentula*	Bacillariophyta	3	2.56E+05	3.22E+08
Cyclotella meneghiniana	Bacillariophyta	1	8.52E+04	3.59E+07
cf. *Cyclotella* sp.	Bacillariophyta	1	8.52E+04	6.10E+07
Cymbella cf. *cistula*	Bacillariophyta	4	4.00E+03	6.31E+07
Cymbella cf. *lanceolata*	Bacillariophyta	1	1.00E+03	7.74E+06
Cymbella cf. *tumida*	Bacillariophyta	1	1.00E+03	8.53E+05
cf. *Diatoma* sp.	Bacillariophyta	6	5.11E+05	1.40E+08
Encyonema cf. *caespitosum*	Bacillariophyta	1	1.00E+03	1.61E+07
Encyonema silesiacum	Bacillariophyta	3	3.00E+03	1.42E+06
cf. *Encyonema* sp.	Bacillariophyta	1	8.52E+04	1.16E+08
Epithemia cf. *argus*	Bacillariophyta	1	1.00E+03	5.00E+06
cf. *Epithemia* sp.	Bacillariophyta	2	1.70E+05	1.79E+09
Epithemia cf. *turgida*	Bacillariophyta	1	8.52E+04	6.82E+08
Fragilaria cf. *crotonensis*	Bacillariophyta	8	2.13E+05	1.74E+08
cf. *Fragilaria* sp.	Bacillariophyta	2	5.32E+04	2.15E+07
Geissleria cf. *dolomitica*	Bacillariophyta	1	8.52E+04	6.22E+06
Geissleria cf. *moseri*	Bacillariophyta	1	8.52E+04	2.39E+07
cf. *Geissleria* sp.	Bacillariophyta	7	7.00E+03	2.07E+07
Gomphonema cf. *angustatum*	Bacillariophyta	1	1.00E+03	1.74E+05
Gomphonema cf. *johnsonii*	Bacillariophyta	1	1.00E+03	3.53E+05

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A1, August 21, 2019 16:15				
Gomphonema cf. *sarcophagus*	Bacillariophyta	1	1.00E+03	6.98E+05
cf. *Gomphonema* sp.	Bacillariophyta	2	5.32E+04	2.19E+07
Gomphonema cf. *sphaerophorum*	Bacillariophyta	1	1.00E+03	2.59E+06
Gyrosigma cf. *acuminatum*	Bacillariophyta	1	8.52E+04	2.17E+08
Navicula cf. *duerrenbergiana*	Bacillariophyta	1	8.52E+04	3.07E+08
Navicula cf. *gregaria*	Bacillariophyta	2	5.32E+04	6.41E+07
Navicula leistikowii	Bacillariophyta	1	8.52E+04	3.49E+07
Navicula cf. *radiosa*	Bacillariophyta	2	2.00E+03	1.66E+07
cf. *Navicula* sp.	Bacillariophyta	3	7.99E+04	1.28E+07
Navicula cf. *splendicula*	Bacillariophyta	3	2.56E+05	2.74E+08
Navicula cf. *viridulacalcis*	Bacillariophyta	1	8.52E+04	4.82E+08
Navigeia cf. *arkonensis*	Bacillariophyta	1	2.66E+04	1.77E+07
Nitzschia cf. *acicularis*	Bacillariophyta	1	1.00E+03	7.71E+05
Nitzschia cf. *amphibia*	Bacillariophyta	1	8.52E+04	7.11E+06
Nitzschia cf. *inconspicua*	Bacillariophyta	1	2.66E+04	1.64E+06
Nitzschia cf. *recta*	Bacillariophyta	1	8.52E+04	1.50E+08
Nitzschia sp.	Bacillariophyta	1	8.52E+04	2.48E+07
Pinnularia cf. *lundii*	Bacillariophyta	1	1.00E+03	7.84E+05
Pinnularia sp.	Bacillariophyta	1	1.00E+03	1.01E+08
cf. *Pinnularia* sp.	Bacillariophyta	1	8.52E+04	1.28E+09
Surirella librile	Bacillariophyta	1	1.00E+03	2.27E+07
Surirella sp.	Bacillariophyta	1	1.00E+03	1.66E+07
Ulnaria ulna	Bacillariophyta	1	1.00E+03	7.66E+06
Actinastrum hantzschii	Chlorophyta	2	1.70E+05	1.15E+07
Carteria sp.	Chlorophyta	1	8.52E+04	1.04E+07
Chloroidium ellipsoideum	Chlorophyta	3	2.56E+05	5.42E+07
Chlorella cf. *vulgaris*	Chlorophyta	10	8.52E+05	7.83E+07
Chlorococcum minutum	Chlorophyta	7	5.96E+05	1.79E+08
cf. *Chlorococcum* sp.	Chlorophyta	1	2.66E+04	4.52E+07
Closteriopsis acicularis	Chlorophyta	1	8.52E+04	1.76E+07
cf. *Coelastrum* sp.	Chlorophyta	10	8.52E+05	2.85E+07
Coelastrum sphaericum	Chlorophyta	6	6.00E+03	8.63E+05
Crucigenia tetrapedia	Chlorophyta	4	4.00E+03	1.63E+06
Desmodesmus bicaudatus	Chlorophyta	2	1.70E+05	4.81E+06
Desmodesmus communis	Chlorophyta	2	1.70E+05	4.81E+06
Dictyosphaerium sp.	Chlorophyta	70	7.00E+04	4.16E+06
cf. *Gloeotila* sp.	Chlorophyta	12	1.20E+04	6.03E+05
Golenkiniopsis sp.	Chlorophyta	1	8.52E+04	1.81E+07

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A1, August 21, 2019 16:15				
Kirchneriella obesa	Chlorophyta	2	5.32E+04	4.91E+06
Monoraphidium arcuatum	Chlorophyta	1	2.66E+04	1.67E+06
Monoraphidium contortum	Chlorophyta	5	4.26E+05	1.45E+07
Monoraphidium griffithii	Chlorophyta	5	4.26E+05	2.01E+07
Monoraphidium minutum	Chlorophyta	20	1.70E+06	3.35E+07
Nautococcus sp.	Chlorophyta	2	1.70E+05	2.45E+08
Oocystis parva	Chlorophyta	1	8.52E+04	1.75E+07
Parapediastrum biradiatum	Chlorophyta	8	6.81E+05	7.46E+07
Pediastrum duplex	Chlorophyta	16	1.36E+06	3.66E+08
Scenedesmus arcuatus	Chlorophyta	8	8.00E+03	5.73E+05
Scenedesmus sp.	Chlorophyta	24	2.04E+06	4.37E+07
Sphaerocystis cf. *planctonica*	Chlorophyta	2	1.70E+05	3.61E+07
Tetradesmus lagerheimii	Chlorophyta	4	4.00E+03	2.82E+05
Cryptomonas erosa	Cryptophyta	6	5.11E+05	2.07E+09
Cryptomonas marssonii	Cryptophyta	3	2.56E+05	7.91E+07
Cryptomonas ovalis	Cryptophyta	15	1.28E+06	1.00E+09
cf. *Cryptomonas* sp.	Cryptophyta	2	1.70E+05	3.11E+07
Plagioselmis nannoplanctica	Cryptophyta	2	1.70E+05	5.75E+07
cf. *Anabaenopsis* sp.	Cyanobacteria	169	1.44E+07	1.15E+09
cf. *Aphanizomenon* sp.	Cyanobacteria	234	1.99E+07	1.78E+09
cf. *Chroococcus* sp.	Cyanobacteria	127	1.08E+07	3.63E+08
Cuspidothrix sp.	Cyanobacteria	253	2.16E+07	1.51E+09
cf. *Cylindrospermopsis* sp.	Cyanobacteria	367	3.13E+07	3.13E+09
Dolichospermum cf. *affine*	Cyanobacteria	143	1.22E+07	1.12E+09
Dolichospermum sp.	Cyanobacteria	101	8.60E+06	3.75E+07
cf. *Eucapsis* sp.	Cyanobacteria	4	3.41E+05	1.43E+06
cf. *Microcystis* sp.	Cyanobacteria	627	6.27E+05	2.10E+07
cf. *Oscillatoria* sp.	Cyanobacteria	194	1.94E+05	9.87E+07
cf. *Planktolyngbya* sp.	Cyanobacteria	700	5.96E+07	5.90E+08
Pseudanabaena cf. *limnetica*	Cyanobacteria	82	6.98E+06	1.10E+07
Pseudanabaena cf. *minima*	Cyanobacteria	4	3.41E+05	2.34E+06
cf. *Pseudanabaena* sp.	Cyanobacteria	82	6.98E+06	1.23E+08
cf. *Raphidiopsis* sp.	Cyanobacteria	33	2.81E+06	5.29E+07
cf. *Romeria* sp.	Cyanobacteria	37	3.15E+06	2.06E+07
Trachelomonas bacillifera	Euglenophyta	1	2.66E+04	8.81E+07
Trachelomonas cf. *intermedia*	Euglenophyta	1	8.52E+04	4.28E+07
Trachelomonas volvocina	Euglenophyta	1	8.52E+04	3.59E+07

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A1, August 21, 2019 16:15				
Dinobryon cf. *bavaricum*	Ochrophyta	1	2.66E+04	2.90E+06
Dinobryon divergens	Ochrophyta	1	1.00E+03	5.85E+05
Goniochloris mutica	Ochrophyta	1	1.00E+03	8.39E+05
Goniochloris sp.	Ochrophyta	1	2.66E+04	7.50E+07
Ceratium hirundinella	Miozoa	4	4.00E+03	2.18E+08
Sample total		3537	2.19E+08	2.30E+10
Site A4, August 21, 2019 18:30				
Achnanthidium sp.	Bacillariophyta	1	3.83E+04	4.01E+06
Acanthoceras sp.	Bacillariophyta	1	3.83E+04	1.21E+08
cf. *Amphora* sp.	Bacillariophyta	1	3.83E+04	1.97E+07
Asterionella formosa	Bacillariophyta	8	4.00E+03	7.25E+06
Aulacoseira alpigena	Bacillariophyta	1	5.00E+02	3.77E+05
Aulacoseira granulata	Bacillariophyta	10	3.83E+05	5.19E+08
Cocconeis cf. *placentula*	Bacillariophyta	1	3.83E+04	1.22E+07
Cyclotella cf. *bodanica*	Bacillariophyta	1	5.00E+02	1.55E+07
Cyclotella meneghiniana	Bacillariophyta	2	7.67E+04	3.23E+07
Epithemia adnata	Bacillariophyta	2	1.00E+03	1.88E+06
Epithemia sp.	Bacillariophyta	1	3.83E+04	1.18E+09
Eunotia cf. *diodon*	Bacillariophyta	1	5.00E+02	2.46E+06
Fragilaria cf. *crotonensis*	Bacillariophyta	13	1.73E+05	4.42E+08
Fragilaria sp.	Bacillariophyta	2	7.67E+04	1.25E+07
cf. *Fragilaria* sp.	Bacillariophyta	9	3.45E+05	2.00E+07
cf. *Geissleria* sp.	Bacillariophyta	1	5.00E+02	5.45E+05
cf. *Gomphoneis* sp.	Bacillariophyta	1	5.00E+02	2.64E+06
Gomphonema cf. *affine*	Bacillariophyta	2	1.00E+03	6.98E+05
Gomphonema sp.	Bacillariophyta	1	5.00E+02	1.90E+06
cf. *Gomphonema* sp.	Bacillariophyta	2	1.00E+03	7.75E+05
Gyrosigma acuminatum	Bacillariophyta	1	5.00E+02	3.90E+06
Melosira cf. *varians*	Bacillariophyta	3	1.15E+05	4.70E+08
Navicula cf. *gregaria*	Bacillariophyta	3	1.50E+03	6.15E+05
Navicula cf. *radiosa*	Bacillariophyta	1	5.00E+02	2.59E+06
Navicula sp.	Bacillariophyta	1	5.00E+02	4.42E+06
cf. *Navicula* sp.	Bacillariophyta	1	3.83E+04	1.19E+07
Navicula cf. *splendicula*	Bacillariophyta	1	5.00E+02	4.82E+05
Navicula vaneei	Bacillariophyta	1	5.00E+02	4.08E+06
Navicula viridulacalcis	Bacillariophyta	1	3.83E+04	1.64E+08

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A4, August 21, 2019 18:30				
Navigeia decussis	Bacillariophyta	1	1.33E+04	1.10E+07
Nitzschia cf. *acicularis*	Bacillariophyta	2	1.00E+03	7.62E+05
cf. *Nitzschia* sp.	Bacillariophyta	1	3.83E+04	9.93E+06
Nitzschia sp.	Bacillariophyta	1	3.83E+04	2.83E+08
Pinnularia cf. *divergens*	Bacillariophyta	1	5.00E+02	1.97E+06
cf. *Placoneis* sp.	Bacillariophyta	1	5.00E+02	1.43E+06
Rhopalodia gibba	Bacillariophyta	2	1.00E+03	4.78E+06
Rhopalodia sp.	Bacillariophyta	1	5.00E+02	1.59E+06
Ulnaria sp.	Bacillariophyta	2	1.00E+03	8.74E+06
Carteria sp.	Chlorophyta	1	1.33E+04	1.62E+06
cf. *Chlamydomonas* sp.	Chlorophyta	1	3.83E+04	2.74E+07
cf. *Chlorella* sp.	Chlorophyta	2	7.67E+04	1.10E+07
Chlorococcum minutum	Chlorophyta	2	7.67E+04	1.63E+07
Coelastrum microporum	Chlorophyta	35	1.75E+04	2.52E+06
Coelastrum sphaericum	Chlorophyta	8	4.00E+03	2.86E+06
Crucigenia tetrapedia	Chlorophyta	20	1.00E+04	6.78E+06
Desmodesmus communis	Chlorophyta	4	2.00E+03	2.51E+04
Kirchneriella irregularis	Chlorophyta	11	4.22E+05	2.30E+07
Monoraphidium arcuatum	Chlorophyta	9	3.45E+05	2.17E+07
Monoraphidium contortum	Chlorophyta	2	7.67E+04	3.62E+06
Monoraphidium minutum	Chlorophyta	11	4.22E+05	8.30E+08
Monoraphidium sp.	Chlorophyta	1	3.83E+04	2.97E+06
cf. *Nautococcus* sp.	Chlorophyta	3	1.15E+05	6.77E+07
Tetradesmus obliquus	Chlorophyta	8	1.06E+05	3.78E+06
Cryptomonas erosa	Cryptophyta	7	3.50E+03	1.39E+07
Cryptomonas marssonii	Cryptophyta	6	2.30E+05	5.27E+08
Cryptomonas ovata	Cryptophyta	1	3.83E+04	9.76E+07
cf. *Cryptomonas* sp.	Cryptophyta	2	7.67E+04	2.44E+07
cf. *Anabaenopsis* sp.	Cyanobacteria	5	1.92E+05	3.49E+07
Aphanizomenon sp.	Cyanobacteria	516	1.98E+07	1.77E+09
cf. *Aphanizomenon* spp.	Cyanobacteria	343	1.31E+07	9.19E+08
Aphanocapsa sp.	Cyanobacteria	88	3.37E+06	1.03E+07
cf. *Chroococcus* spp.	Cyanobacteria	79	3.03E+06	2.78E+08
Dolichospermum cf. *planctonicum*	Cyanobacteria	181	6.94E+06	2.92E+09
cf. *Dolichospermum* sp.	Cyanobacteria	296	1.13E+07	1.04E+09
Eucapsis microscopica	Cyanobacteria	100	3.83E+06	6.77E+06
cf. *Eucapsis* sp.	Cyanobacteria	27	1.03E+06	1.46E+07

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A4, August 21, 2019 18:30				
Microcystis sp.	Cyanobacteria	875	4.37E+05	2.09E+07
cf. *Phormidium* sp.	Cyanobacteria	990	4.95E+05	1.06E+07
cf. *Planktolyngbya* sp.	Cyanobacteria	203	7.78E+06	1.11E+08
Pseudanabaena limnetica	Cyanobacteria	18	6.90E+05	6.50E+06
Pseudanabaena minima	Cyanobacteria	136	5.21E+06	2.65E+07
Pseudanabaena sp.	Cyanobacteria	320	1.23E+07	8.43E+07
cf. *Pseudanabaena* sp.	Cyanobacteria	93	3.56E+06	5.08E+07
cf. *Romeria* sp.	Cyanobacteria	22	8.43E+05	2.98E+06
Synechococcus sp.	Cyanobacteria	13	4.98E+05	1.32E+07
Woronichinia sp.	Cyanobacteria	70	3.50E+04	7.48E+05
Trachelomonas cf. *volvocinopsis*	Euglenophyta	4	2.00E+03	4.88E+06
cf. *Chromulina* sp.	Ochrophyta	1	3.83E+04	1.28E+06
Ceratium hirundinella	Miozoa	1	5.00E+02	2.76E+07
Pseudostaurastrum hastatum	Xanthophyta	1	3.83E+04	4.99E+07
Sample total		4602	9.82E+07	1.25E+10
Site A5, August 21, 2019 17:05				
Aulacoseira sp.	Bacillariophyta	13	5.98E+05	7.15E+08
Cocconeis cf. *placentula*	Bacillariophyta	4	1.60E+03	2.61E+06
Epithemia sp.	Bacillariophyta	2	8.00E+02	1.46E+06
Epithemia cf. *turgida*	Bacillariophyta	2	9.20E+04	1.40E+09
cf. *Fragilaria* sp.	Bacillariophyta	10	4.00E+03	1.31E+06
Gomphonema sp.	Bacillariophyta	1	4.00E+02	3.50E+05
Navicula cf. *phyllepta*	Bacillariophyta	2	8.00E+02	7.24E+05
Navicula cf. *radiosa*	Bacillariophyta	1	4.60E+04	8.70E+07
cf. *Navicula* sp.	Bacillariophyta	3	1.20E+03	4.10E+05
Nitzschia sp.	Bacillariophyta	2	8.00E+02	4.53E+05
cf. *Synedra* sp.	Bacillariophyta	2	8.00E+02	5.16E+06
Ulnaria sp.	Bacillariophyta	1	4.60E+04	9.66E+08
cf. *Chlorella* sp.	Chlorophyta	3	1.38E+05	1.27E+07
Chlorococcum minutum	Chlorophyta	20	9.20E+05	1.32E+08
Coelastrum microporum	Chlorophyta	18	7.20E+03	1.53E+06
cf. *Coelastrum* sp.	Chlorophyta	25	1.00E+04	2.99E+06
Crucigenia tetrapedia	Chlorophyta	4	1.60E+03	3.24E+05
Kirchneriella obesa	Chlorophyta	1	4.00E+02	6.20E+04
cf. *Kirchneriella* sp.	Chlorophyta	1	4.00E+02	4.24E+04
Monoraphidium arcuatum	Chlorophyta	1	4.60E+04	2.89E+06
Monoraphidium contortum	Chlorophyta	3	1.38E+05	4.68E+06

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A5, August 21, 2019 17:05				
Monoraphidium griffithii	Chlorophyta	2	8.00E+02	9.94E+04
Monoraphidium minutum	Chlorophyta	13	5.98E+05	1.18E+07
cf. *Nautococcus* sp.	Chlorophyta	2	8.00E+02	9.20E+05
Nephrocytium limneticum	Chlorophyta	4	1.60E+03	1.25E+05
Oocystis cf. *naegelli*	Chlorophyta	1	4.00E+02	4.77E+05
Oocystis parva	Chlorophyta	4	1.60E+03	1.26E+05
cf. *Scotiellopsis* sp.	Chlorophyta	1	4.60E+04	1.24E+08
Tetradesmus obliquus	Chlorophyta	24	1.10E+06	2.36E+07
Tetraedron minimum	Chlorophyta	1	4.60E+04	3.65E+07
Chroomonas coerulea	Cryptophyta	1	4.60E+04	8.57E+06
Cryptomonas erosa	Cryptophyta	4	1.84E+05	2.83E+08
Cryptomonas marssonii	Cryptophyta	4	1.84E+05	1.78E+08
Cryptomonas ovata	Cryptophyta	7	3.22E+05	5.29E+07
cf. *Cryptomonas* sp.	Cryptophyta	1	4.00E+02	2.68E+05
Plagioselmis nannoplanctica	Cryptophyta	13	5.98E+05	2.02E+08
cf. *Anabaenopsis* sp.	Cyanobacteria	143	6.58E+06	1.20E+09
Aphanizomenon sp.	Cyanobacteria	218	1.00E+07	1.83E+09
cf. *Aphanizomenon* sp.	Cyanobacteria	49	2.25E+06	1.79E+08
Aphanocapsa sp.	Cyanobacteria	10	4.60E+05	1.93E+06
cf. *Chroococcus* sp.	Cyanobacteria	61	2.81E+06	2.58E+08
cf. *Cuspidothrix* sp.	Cyanobacteria	35	1.61E+06	4.15E+07
cf. *Cylindrospermopsis* sp.	Cyanobacteria	33	1.52E+06	1.21E+08
Dolichospermum planctonicum	Cyanobacteria	412	1.90E+07	7.98E+09
Dolichospermum sp.	Cyanobacteria	341	1.57E+07	5.26E+08
Eucapsis microscopica	Cyanobacteria	34	1.56E+06	4.78E+06
cf. *Phormidium* sp.	Cyanobacteria	166	7.64E+06	3.76E+08
cf. *Planktolyngbya* sp.	Cyanobacteria	1980	9.11E+07	5.95E+08
Pseudanabaena minima	Cyanobacteria	18	8.28E+05	5.69E+06
Pseudanabaena sp.	Cyanobacteria	187	8.60E+06	8.10E+07
cf. *Pseudanabaena* spp.	Cyanobacteria	294	1.35E+07	1.93E+08
Romeria sp.	Cyanobacteria	267	1.23E+07	1.75E+08
Trachelomonas cf. *pulchella*	Euglenophyta	6	2.40E+03	5.09E+06
cf. *Trachelomonas* sp.	Euglenophyta	10	4.00E+03	6.79E+06
Trachelomonas volvocina	Euglenophyta	1	4.00E+02	2.86E+05
Ochromonas variabilis	Ochrophyta	3	1.38E+05	2.57E+07
Ceratium furcoides	Miozoa	2	8.00E+02	2.77E+07
Ceratium hirundinella	Miozoa	4	1.84E+05	8.91E+09

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A5, August 21, 2019 17:05				
cf. *Gyrodinium* sp.	Miozoa	4	1.60E+03	1.15E+06
Peridinium bipes	Miozoa	2	8.00E+02	3.47E+07
Peridinium cf. *cinctum*	Miozoa	3	1.20E+03	8.73E+07
Peridinium lomnickii	Miozoa	2	8.00E+02	6.76E+06
Sample total		4486	2.01E+08	2.69E+10
Site A1, October 17, 2019 12:20				
Achnanthidium sp.	Bacillariophyta	1	1.53E+04	4.88E+06
Asterionella formosa	Bacillariophyta	41	6.29E+05	3.04E+08
Aulacoseira alpigena	Bacillariophyta	14	2.15E+05	3.99E+07
Aulacoseira sp.	Bacillariophyta	3	4.60E+04	2.52E+07
cf. *Aulacoseira* sp.	Bacillariophyta	19	6.33E+03	2.15E+06
Cocconeis cf. *placentula*	Bacillariophyta	1	1.53E+04	2.00E+07
Cyclotella meneghiniana	Bacillariophyta	1	1.53E+04	1.83E+07
Cyclotella quillensis	Bacillariophyta	4	1.33E+03	9.65E+06
cf. *Cyclotella* sp.	Bacillariophyta	12	1.84E+05	3.96E+07
Cyclotella sp.	Bacillariophyta	28	4.29E+05	5.13E+08
Cymbella cf. *mexicana*	Bacillariophyta	2	6.67E+02	1.61E+07
Cymbella cf. *tumida*	Bacillariophyta	2	3.07E+04	2.13E+07
Encyonema cf. *minutum*	Bacillariophyta	4	6.13E+04	2.28E+07
Encyonema cf. *silesiacum*	Bacillariophyta	1	3.33E+02	3.20E+05
Epithemia sp.	Bacillariophyta	1	3.33E+02	2.10E+06
Fragilaria cf. *crotonensis*	Bacillariophyta	4	1.33E+03	1.06E+06
Fragilaria sp.	Bacillariophyta	1	8.87E+03	1.03E+06
cf. *Fragilaria* sp.	Bacillariophyta	1	8.87E+03	4.04E+06
Gomphonema cf. *affinopsis*	Bacillariophyta	1	3.33E+02	2.05E+05
Gomphonema cf. *parvulum*	Bacillariophyta	4	1.33E+03	1.97E+05
Gomphonema sp.	Bacillariophyta	2	3.07E+04	1.28E+07
cf. *Gomphonema* sp.	Bacillariophyta	1	3.33E+02	8.31E+05
Gyrosigma sp.	Bacillariophyta	1	8.87E+03	1.62E+08
cf. *Mayamaea* sp.	Bacillariophyta	1	1.53E+04	3.14E+06
Melosira varians	Bacillariophyta	29	9.67E+03	3.87E+06
cf. *Meridion* sp.	Bacillariophyta	1	3.33E+02	2.05E+05
cf. *Microcostatus* sp.	Bacillariophyta	1	1.53E+04	1.16E+06
Navicula cf. *phyllepta*	Bacillariophyta	3	1.00E+03	1.35E+06
Navicula cf. *radiosa*	Bacillariophyta	2	6.67E+02	2.33E+06
Navicula cf. *salinarum*	Bacillariophyta	2	6.67E+02	4.64E+05
Navicula cf. *slesvicensis*	Bacillariophyta	1	3.33E+02	7.96E+05

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A1, October 17, 2019 12:20				
cf. *Navicula* sp.	Bacillariophyta	2	6.67E+02	1.33E+06
Navicula cf. *striolata*	Bacillariophyta	1	3.33E+02	2.47E+06
Navigeia cf. *paludosa*	Bacillariophyta	1	1.53E+04	4.19E+06
Nitzschia cf. *acicularis*	Bacillariophyta	1	1.53E+04	3.13E+06
Nitzschia cf. *inconspicua*	Bacillariophyta	2	3.07E+04	1.53E+06
Nitzschia cf. *paleacea*	Bacillariophyta	2	6.67E+02	6.34E+04
Nitzschia cf. *recta*	Bacillariophyta	1	3.33E+02	7.94E+04
Nitzschia cf. *regula*	Bacillariophyta	1	3.33E+02	4.86E+05
Nitzschia cf. *sigma*	Bacillariophyta	1	3.33E+02	9.60E+05
cf. *Nitzschia* sp.	Bacillariophyta	1	1.53E+04	3.68E+06
Placogeia cf. *similis*	Bacillariophyta	1	1.53E+04	8.98E+06
Surirella cf. *robusta*	Bacillariophyta	1	3.33E+02	1.80E+07
cf. *Synedra* sp.	Bacillariophyta	2	3.07E+04	1.29E+08
Tabellaria fenestrata	Bacillariophyta	1	8.87E+03	6.68E+06
cf. *Ulnaria* sp.	Bacillariophyta	1	1.53E+04	2.50E+08
Ulnaria ulna	Bacillariophyta	1	3.33E+02	1.09E+06
cf. *Cosmarium* sp.	Charophyta	1	1.53E+04	1.55E+06
cf. *Chlamydomonas* sp.	Chlorophyta	2	3.07E+04	1.29E+07
cf. *Chlorella* sp.	Chlorophyta	2	3.07E+04	4.41E+06
cf. *Closteriopsis* sp.	Chlorophyta	1	3.33E+02	1.90E+05
cf. *Coelastrum* sp.	Chlorophyta	15	5.00E+03	1.68E+05
Crucigenia tetrapedia	Chlorophyta	8	1.23E+05	1.66E+07
Desmodesmus bicaudatus	Chlorophyta	4	3.55E+04	6.91E+05
Desmodesmus communis	Chlorophyta	2	1.77E+04	6.30E+05
Monoraphidium arcuatum	Chlorophyta	2	3.07E+04	2.29E+06
Monoraphidium contortum	Chlorophyta	6	9.20E+04	4.37E+06
Monoraphidium griffithii	Chlorophyta	1	1.53E+04	1.93E+06
cf. *Muriella* sp.	Chlorophyta	5	1.67E+03	2.83E+06
cf. *Scenedesmus* sp.	Chlorophyta	5	7.67E+04	5.11E+06
Schroederia setigera	Chlorophyta	1	3.33E+02	4.03E+05
Tetradesmus obliquus	Chlorophyta	4	6.13E+04	1.19E+06
cf. *Synura* sp.	Chrysophyta	4	6.13E+04	2.99E+07
Chroomonas coerulea	Cryptophyta	8	1.23E+05	2.83E+07
Cryptomonas erosa	Cryptophyta	36	5.52E+05	1.08E+09
Cryptomonas marssonii	Cryptophyta	71	1.09E+06	5.10E+08
Cryptomonas ovata	Cryptophyta	1	1.53E+04	9.03E+06
Cryptomonas sp.	Cryptophyta	2	3.07E+04	4.39E+07

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A1, October 17, 2019 12:20				
cf. *Cryptomonas* sp.	Cryptophyta	3	4.60E+04	2.50E+07
Plagioselmis nannoplanctica	Cryptophyta	41	6.29E+05	2.57E+08
cf. *Aphanizomenon* sp.	Cyanobacteria	12	1.84E+05	1.46E+07
cf. *Chroococcus* sp.	Cyanobacteria	21	3.22E+05	2.96E+07
cf. *Cylindrospermopsis* sp.	Cyanobacteria	72	1.10E+06	2.53E+08
Dolichospermum sp.	Cyanobacteria	21	7.00E+03	1.49E+06
cf. *Planktolyngbya* sp.	Cyanobacteria	52	7.97E+05	5.21E+06
Trachelomonas cf. *caudata*	Euglenophyta	1	3.33E+02	6.02E+05
Trachelomonas cf. *volvocina*	Euglenophyta	2	3.07E+04	5.87E+07
Dinobryon cf. *divergens*	Ochrophyta	4	6.13E+04	2.51E+07
cf. *Gymnodinium* sp.	Miozoa	4	1.33E+03	1.13E+07
Peridinium cf. *lomnickii*	Miozoa	1	3.33E+02	5.13E+06
cf. *Peridinium* sp.	Miozoa	2	6.67E+02	2.21E+06
Sample total		626	7.49E+06	4.12E+09
Site A4, October 17, 2019 10:55				
Achnanthidium exiguum	Bacillariophyta	1	1.80E+05	2.47E+07
Aulacoseira granulata	Bacillariophyta	39	5.20E+04	1.14E+08
Aulacoseira sp.	Bacillariophyta	2	3.61E+05	5.17E+08
Cocconeis cf. *placentula*	Bacillariophyta	2	2.67E+03	2.41E+06
Cyclotella bodanica	Bacillariophyta	2	3.61E+05	1.82E+10
Cyclotella meneghiniana	Bacillariophyta	3	5.41E+05	6.46E+08
Cymbella cf. *cistula*	Bacillariophyta	3	4.00E+03	1.15E+08
cf. *Diatoma* sp.	Bacillariophyta	13	1.73E+04	1.06E+07
cf. *Encyonema* sp.	Bacillariophyta	1	1.80E+05	2.26E+08
Epithemia sp.	Bacillariophyta	3	4.00E+03	6.61E+07
Fragilaria capucina	Bacillariophyta	1	1.33E+03	4.56E+05
Fragilaria cf. *crotonensis*	Bacillariophyta	15	2.00E+04	4.94E+07
Gomphonema cf. *affinopsis*	Bacillariophyta	2	2.67E+03	4.67E+06
Gomphonema cf. *exilissimum*	Bacillariophyta	1	1.33E+03	1.27E+07
cf. *Gyrosigma* sp.	Bacillariophyta	1	1.33E+03	5.81E+06
Navicula cf. *capitatoradiata*	Bacillariophyta	1	1.33E+03	1.65E+06
Navicula cf. *gregaria*	Bacillariophyta	1	1.80E+05	2.32E+08
Navicula cf. *radiosa*	Bacillariophyta	2	2.67E+03	1.31E+07
cf. *Navicula* sp.	Bacillariophyta	1	1.33E+03	1.10E+06
Navicula sp.	Bacillariophyta	2	2.67E+03	5.03E+07
Nitzschia spp.	Bacillariophyta	3	5.41E+05	1.00E+08
Stephanodiscus sp.	Bacillariophyta	1	1.33E+03	5.70E+07

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A4, October 17, 2019 10:55				
Carteria cf. *kelbsii*	Chlorophyta	2	2.67E+03	1.77E+07
Chlorella vulgaris	Chlorophyta	5	9.02E+05	8.29E+07
Chloroidium ellipsoideum	Chlorophyta	3	5.41E+05	2.52E+07
cf. *Closterium* sp.	Chlorophyta	1	1.80E+05	2.51E+08
cf. *Coccomyxa* sp.	Chlorophyta	2	3.61E+05	1.02E+07
Keratococcus dispar	Chlorophyta	2	3.61E+05	4.53E+06
Kirchneriella cf. *irregularis*	Chlorophyta	51	9.20E+06	2.23E+08
Kirchneriella obesa	Chlorophyta	1	1.33E+03	1.42E+05
Monoraphidium arcuatum	Chlorophyta	1	1.80E+05	1.40E+07
Monoraphidium contortum	Chlorophyta	3	5.41E+05	2.55E+07
cf. *Synura* sp.	Chrysophyta	1	3.55E+04	1.78E+07
Cryptomonas erosa	Cryptophyta	3	5.41E+05	1.33E+09
Cryptomonas marssonii	Cryptophyta	6	1.08E+06	8.63E+08
Cryptomonas cf. *ovata*	Cryptophyta	2	2.67E+03	3.23E+07
Plagioselmis nannoplanctica	Cryptophyta	13	2.34E+06	1.59E+09
Aphanizomenon sp.	Cyanobacteria	218	3.93E+07	3.93E+09
cf. *Chroococcus* sp.	Cyanobacteria	394	7.11E+07	3.39E+09
Dolichospermum sp.	Cyanobacteria	41	5.47E+04	7.86E+06
cf. *Phormidium* sp.	Cyanobacteria	107	3.80E+06	3.46E+08
Planktolyngbya sp.	Cyanobacteria	30	5.41E+06	5.10E+07
Pseudanabaena sp.	Cyanobacteria	122	2.20E+07	1.55E+09
cf. *Synechococcus* sp.	Cyanobacteria	5	9.02E+05	1.28E+07
Ochromonas cf. *variabilis*	Ochrophyta	1	1.80E+05	6.82E+07
Sample total		1114	1.61E+08	3.43E+10
Site A5, October 17, 2019 11:55				
Aulacoseira cf. *granulata*	Bacillariophyta	147	4.67E+04	1.12E+08
Cyclotella bodanica	Bacillariophyta	12	3.81E+03	2.49E+08
Cyclotella meneghiniana	Bacillariophyta	3	9.52E+02	2.32E+06
Cyclotella sp.	Bacillariophyta	1	3.17E+02	1.61E+06
cf. *Cyclotella* sp.	Bacillariophyta	1	4.06E+04	1.71E+07
Cymbella cf. *cistula*	Bacillariophyta	1	3.17E+02	2.75E+06
Melosira sp.	Bacillariophyta	1	3.17E+02	4.21E+05
Melosira cf. *varians*	Bacillariophyta	49	1.56E+04	4.35E+06
cf. *Ulnaria* sp.	Bacillariophyta	1	3.17E+02	1.17E+07
Botryococcus braunii	Chlorophyta	72	2.29E+04	1.11E+07
cf. *Scenedesmus* sp.	Chlorophyta	56	2.27E+06	5.22E+07
Cryptomonas erosa	Cryptophyta	2	6.35E+02	6.38E+05

Table A1. Phytoplankton identification, enumeration, density, and biovolume from three cyanobacterial bloom sites in Kabetogama Lake, Voyageurs National Park. (continued)

Genus	Division	Tally	Density (cells/L)	Biovolume (mm^3/L)
Site A5, October 17, 2019 11:55				
Cryptomonas marssonii	Cryptophyta	1	4.06E+04	1.51E+07
Cryptomonas ovata	Cryptophyta	1	3.17E+02	1.18E+05
cf. *Anabaenopsis* sp.	Cyanobacteria	15	6.08E+05	5.66E+07
Aphanizomenon sp.	Cyanobacteria	1463	5.93E+07	5.52E+09
Aphanocapsa sp.	Cyanobacteria	223	7.08E+04	2.97E+05
cf. *Chroococcus* sp.	Cyanobacteria	224	9.09E+06	8.35E+08
Dolichospermum cf. *planctonicum*	Cyanobacteria	145	4.60E+04	3.30E+07
Dolichospermum sp.	Cyanobacteria	89	3.61E+06	6.73E+08
Microcystis sp.	Cyanobacteria	7910	2.51E+06	2.31E+08
cf. *Phormidium* sp.	Cyanobacteria	534	2.17E+07	1.49E+09
Planktolyngbya sp.	Cyanobacteria	27	1.10E+06	1.03E+07
Pseudanabaena cf. *limnetica*	Cyanobacteria	4	1.62E+05	1.53E+06
Pseudanabaena sp.	Cyanobacteria	535	2.17E+07	1.10E+08
Woronichinia sp.	Cyanobacteria	1673	5.31E+05	2.13E+07
Sample total		13190	1.23E+08	9.46E+09

[cells/L, cells per liter; mm^3/L, cubic micrometers per liter]